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13. ABSTRACT *(Maximum 200 Words)*

This project investigated experimental models with lab animals, which can be used to identify possible causes and therapies for the "Gulf War Syndrome." Some personnel who served in the 1991 Persian Gulf war have reported persistent problems that are dominated by cognitive, neurological and respiratory complaints. In order to clarify the causal factors, rats were evaluated as models of low level exposures to a potential causative agent: oral exposure to anti cholinesterase medication pyridostigmine (PB), and psychological stress resulting from exposure to novel stimuli (Stress). There have been few published experiments with animals receiving dynamic inhalation exposures to chemical warfare agent, and none which have studied combined exposures to such agents + PB + Stress. The health effects of these 3 variables combined may be different than the effects of any one exposure. Quantitative models such as these will permit future studies with human subjects to be more focused and mechanistically driven and future studies with animal models to develop better therapies for poisoning and/or stress-related illnesses.

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Gulf War, Gulf War syndrome, organophosphate, anti-cholinesterase, behavioral disorders, pulmonary disorders, neurotoxicity, brain function, animal models

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FOREWORD

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X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Hugh L. Evans_ November 26, 2001
PI - Signature Date

Table of Contents

1. Evans, HL, Bushnell PJ, et al. Fundam. Appl, Toxicol. 6:721-732, 1986.
2. Wolthuis, OL and Vanwersch, AP. Fundam. Appl. Toxicol. 4:s195-s208, 1984.
3. Hoy, JB, Cody, BA, et al., Pharmacol. Biochem. Behav. 63:401-406, 1999.
4. van Haaren, F., Haworth, SC., et al., Pharmacol. Biochem. Behav. 68:81-85, 2001

Key Research Accomplishments. The accomplishments under this contract are summarized with respect to the five original parts of the Statement of Work.

1. This project aimed to provide rigorous experimental models of the persistent signs and symptoms observed in humans known as the Gulf War Syndrome (GWS). We planned to measure in rats and monkeys the same behavioral, neurological and electrophysiological sequelae seen in humans with the GWS. These animal models with multiple end-points could then be compared directly in human and lab animals. We began experiments with rats to examine the influence of psychological stress on the effects of chemical exposures like those of the Gulf War veterans. Research equipment to measure behavioral and pulmonary responses has been upgraded to Windows 2000. A computer consultant prepared queries which can organize the data using MS-ACCESS. The biostatistician has advised on the sample size, statistical power, archiving of database, methods to transfer data files from MS-ACCESS in our lab to the Biostatistics facility for statistical analyses using SAS.

The results of the first study showed that our behavioral test system equaled the sensitivity to pyridostigmine bromide (PB; >99% purity, Sigma Chemical Co., St. Louis MO.) of other methods of studying behavior of rats (Wolthuis and Vanwersch, 1984). Each adult male F334 rat (weight range 282 — 357 g) received oral gavage with zero PB (physiological saline), 10 mg/kg PB (5 mg/ml) or 30 mg/kg PB (15 mg/ml) 30 min before being removed from their Lucite home cage and placed in an unfamiliar Lucite cage with fresh saw dust on the floor. The oral route of exposure closely models the route of PB exposure in Gulf War veterans. The experiment was

conducted in a lighted room during the lighted part of the daily light-dark cycle. A computerized Photocell Activity System for Cage Rack (San Diego Instruments, San Diego, CA) was used for non-invasive, real-time measurement of behavior. This type of system was originally developed and validated in this lab (Evans et al., 1986; see APPENDICES). Rats were removed from their home cage in which they lived in groups of 2, and placed, individually, in a larger, unfamiliar cage with clean sawdust as bedding. Locomotion, fine movements and rearing were recorded at intervals of 5 min for a total test session of 60 min. The dose-response function for PB resembled an inverted U, with the significant increases in fine movement, locomotion and rearing after 10 mg/kg PB. After 30 mg/kg, some behavioral indices were decreased to less than that of the control group. Figure 1 shows the effect of PB on the total amount of rearing behavior during a 60-minute test session. A one-way ANOVA showed that PB dose variable was statistically significant (df=2,7 P=0.0002). Figure 2 shows the temporal pattern of rearing at 5-min intervals for the same rats. Control rats exhibited the expected habituation to the unfamiliar cage, indicated by a decline in amount of rearing after 10 — 20 min. There was a second episode of activity at 25 mins, which declined to near-zero by 30 mins. Rats given 30 mg/kg remained inactive after 15 min, suggesting lethargy and/or toxicity, possibly resulting from accumulation of excessive amounts of acetylcholinne at neuromuscular junctions. In contrast, rats given 10 mg/kg PB exhibited increased rearing which lasted without pause for 30 min after being placed in the unfamiliar cage. The rats given 10 mg/kg PB also exhibited significantly more fine movements and significantly more ambulations than the control rats.

Other laboratories have reported only reduced amounts of locomotor behavior of rats following exposure to PB (Hoy et al., 1999; van Haaren et al., 2001 see APPENDICES). The present results suggest that a relatively low, non-toxic dose of PB (10 mg/kg) can render the rat

hyper-reactive when confronted with the mild stress of an unfamiliar cage. This might provide a model for altered response to stressful stimuli, which is believed to be a component of the Gulf War Syndrome. The higher dose of PB (30 mg/kg) caused a reduction in all 3 behavioral categories, which is interpreted as a toxic effect involving cholinergic dysfunction.

Several stress methods (restraint for one hour or placement in an unfamiliar cage) were studied to learn how they could be used in conjunction with nose-only inhalation exposure methods. Preliminary studies by the present researchers have found that the polycarbonate plastic tube, used to restrain rats during nose-only inhalation exposures, does not produce noticeable habituation. Therefore, a similar device could be used for the restraint-stress variable in the proposed studies. Current studies did not progress far enough to indicate one preferred protocol over alternatives.

2. Our experiments were planned to employ realistic routes of exposure to potential causative agents, including inhalation of organophosphate (OP) nerve agents (Soman, SO; Sarin, SA) and/or oral exposure to prophylactic medicines such as pyridostigmine bromide (PB, described above in part 1). We accomplished renovations to meet the high standards of security and safety demanded by the Army for research with OP nerve agents. Secure storage for chemical warfare agent was prepared in room 177. Figure 3 (see APPENDICES) shows the survey of security, with entrances to the ground floor controlled by ID card and keys. A facility in room 169 was renovated for controlled exposures of lab animals to a highly hazardous chemical agent by inhalation. Figure 4 shows exits which can be used in the event of a spill in either room 169 or 177.

We developed new state-of-the art hardware and software system for real-time measurement of chemical agent in air. The system provides two measurements simultaneously: (1) trace concentrations (below the TWA) in the laboratory air for monitoring occupational safety of those working on this research, and for confirming the integrity of our air exhaust filters. (2) Higher concentrations, inside the inhalation exposure chamber, needed for scientific purposes to document the level of exposure of the experimental animals. Measurement of higher concentrations of OP required development of a working collaboration with the Lovelace Respiratory Research Institute (Albuquerque, NM), which has been studying inhalation exposure of sarin by rats, and the O-I-Analytical Technical Support Group (Pelham, AL), the manufacture of the MINICAMS hardware and software system. Figure 5 (see APPENDICES) shows a schematic diagram of the exhaust flows from the lab, room 169. Figure 6 shows a schematic diagram of the air supply and chemical sampling for the lab, room 169.

These preparations for the start of experiments required much more time and effort than we had estimated. The RFA did not indicate extraordinary measures would be required. The Contract Clauses stating safety and security requirements were delivered after the award. None of the research staff available for this project have participated in GLP-type research with high security requirements. The technician from Environmental Services who was assisting the PI has transferred to a new job and could not be replaced. In light of the serious concerns about the adequacy of our national security after the disasters of Sept. 11, 2001, it appears prudent that we chose a cautious, methodical approach in preparing a detailed Facilities Safety Plan of several hundred pages. Figures 3 through 6 are examples. After the start of the project, a site visit by the Army indicated that the chemical exposure lab should be relocated from room 128 to room 169 so as to provide better security and safety. Planning, movement of equipment and upgrade has taken

time. A research technician, recruited to assist with this work, resigned to take a position in industry after 2 months. It has proven difficult to staff this project with highly-qualified research and support personnel. Thus, it proved to be a daunting task to implement the Army's safety and security regulations in an academic institution.

In spite of these obstacles, we made progress in developing a scheme for the first real-time measurement of chemical warfare agent during inhalation experiments. After the start of the project, the Army informed us of the existence of the GC device known as MINICAMS which is the method best suited for near real time measurements of chemical warfare agent in air. We began the process of specifying the new equipment with the manufacturer, requesting budgeting approval from the Army, installation of the new equipment and training staff in its use. We learned from our collaborators at Lovelace Institute that an earlier version of the MINICAMS could not measure higher concentrations needed for animal experiments. This shortcoming created a delay while the manufacturer modified the equipment design and usage protocol. This instrumentation became a major, but unplanned, undertaking of the project. The work did not progress to experiments with inhaled Sarin due to delays for modifications of equipment and in staffing the vacancies for research and environmental services technicians.

3. The project would have studied delayed effects and long-term consequences of chemical exposures, which are below the level producing acute effects. The powerful prospective (repeated measures) experimental design would provide a pre-exposure baseline of each end point so that each animal can serve as its own control. We were not able to do this because of difficulties in implementing the handling of Sarin and the difficulties in renovating the animal housing facilities to accommodate new regulations for primates. A further delay was attributed to the extremely

limited supply of primates at the present time. Collaborative arrangements were prepared so that handling of Sarin and work with primates could be done at Lovelace Respiratory Research Institute and at the National Center for Toxicological Research (Jefferson, AK). However, the sub-contracts had not yet been completed by the business offices of the respective institutions by the time the project was ended.

4. The exposure to the chemicals would be indexed by well-established biomarkers of exposure to OPs (red blood cell cholinesterase, RCCh, and neuropathy target esterase, NTE). This was not done because the exposures had not yet taken place.

5. It was hypothesized that stress may alter responsiveness to environmental stimuli, and that long-lasting, subclinical effects of the GWS may result from the combined effects of chemical agents and stress. We planned to expose animals to chemicals and to the stress of an unfamiliar environment, to model the experience of the veterans during the Gulf War. Studies with rats are described above in part 1. Studies with primates were not done due to limited availability of primates and the need to change of laboratory sites for primate studies.

Planning for Rapid Response to Bio-terrorism.

The disaster of September 11, 2001, at the World Trade Center indicated that New York City was an area targeted by terrorists. This indicates the need for a stronger infrastructure and for careful planning of public safety and security. New York University convened a Center for Health Information and Preparedness (CHIP), which would assemble information, expertise and facilities that could be drawn upon quickly, should the University be confronted by bio-terrorism. Dr. Evans

became chair of a subcommittee on this CHIP committee, and was able to contribute information on chemical warfare agents, derived in part from his experience with this research project. The MINICAMS GCs were included in a list of equipment that could be made available in the event of a mass terrorism incident.

Conclusions and General Summary.

There has been no published, peer-reviewed research with dynamic inhalation exposures to nerve agents. The reason that few labs have attempted this research and that none have published thus far is the daunting nature of the task. Add to this the special needs of research with non-human primates, and one sees that this is a complex and difficult undertaking in which few labs have the desire and capability. None others have succeeded to date. In this context, we made reasonable progress toward an intelligent and imaginative scheme for experiments to provide the needed information.

APPENDICES

Figure Legends:

1. The effect of pyridostigmine bromide administered *p.o.* (PB) on the amount of rearing behavior of rats for 60 min after being placed into an unfamiliar cage. The results reflect an inverted-U dose-effect curve, with enhanced rearing at 10 mg/kg and reduced rearing at 30 mg/kg. Each data point represents one rat. The curve is drawn through the mean of each group. N = 3 for the control and the 10 mg/kg groups. N=4 for 30 mg/kg.

2. The influence of PB upon the habituation pattern of rearing by rats during the 60 min after placement in an unfamiliar cage. Animals and doses same as shown in Fig. 1.

3. Security system showing control of entry to the ground floor of the complex. In addition, access to the lab in room 169 and to chemical storage in room 177 was accessible only by key issued to senior research staff.

4. Safety plan showing the exits from the ground floor of the complex, which contains the lab in room 169 and chemical storage in room 177.

5. Schematic diagram showing exhaust flows in room 169 which carry the Chemical Warfare Agent through measurement devices (Minicams #1 and #2), the glove box and animal exposure chamber, and exhaust above the roof.

6. Schematic diagram showing sampling of air supply in room 169, both for occupational safety monitoring (Minicams #2) and for control of the experimental exposure chamber (Minicams #1).

References to the literature;

1. Evans, HL, Bushnell PJ, et al. *Fundam. Appl, Toxicol.* 6:721-732, 1986.

2. Wolthuis, OL and Vanwersch, AP. *Fundam. Appl. Toxicol.* 4:s195-s208, 1984.

3. Hoy, JB, Cody, BA, et al., *Pharmacol. Biochem. Behav.* 63:401-406, 1999.

4. van Haaren, F., Haworth, SC., et al., *Pharmacol. Biochem. Behav.* 68:81-85, 2001

Figure 1

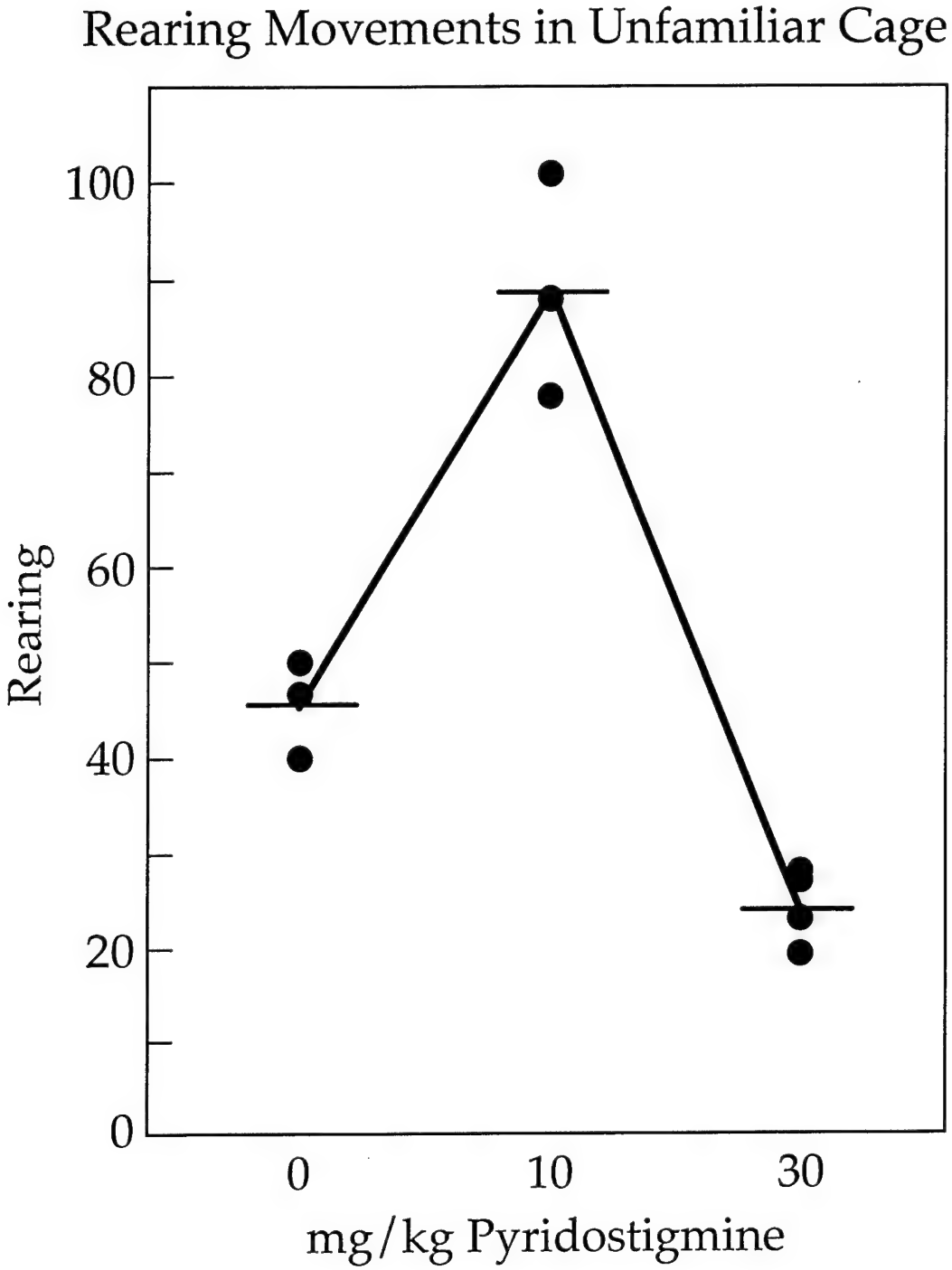
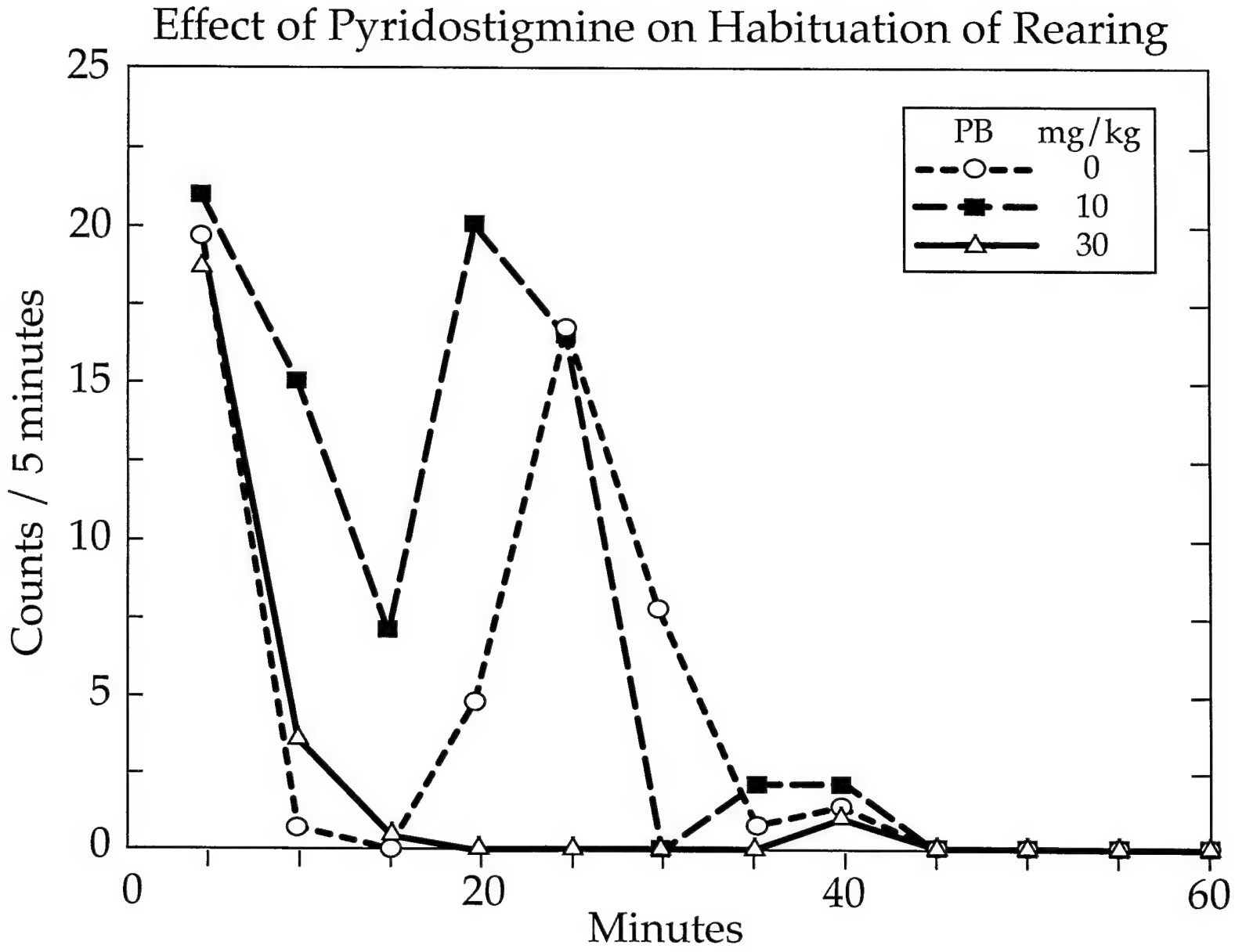

Rearing Movements in Unfamiliar Cage
Rearing
100
80
60
40
20
0
0
10
30
mg/kg Pyridostigmine

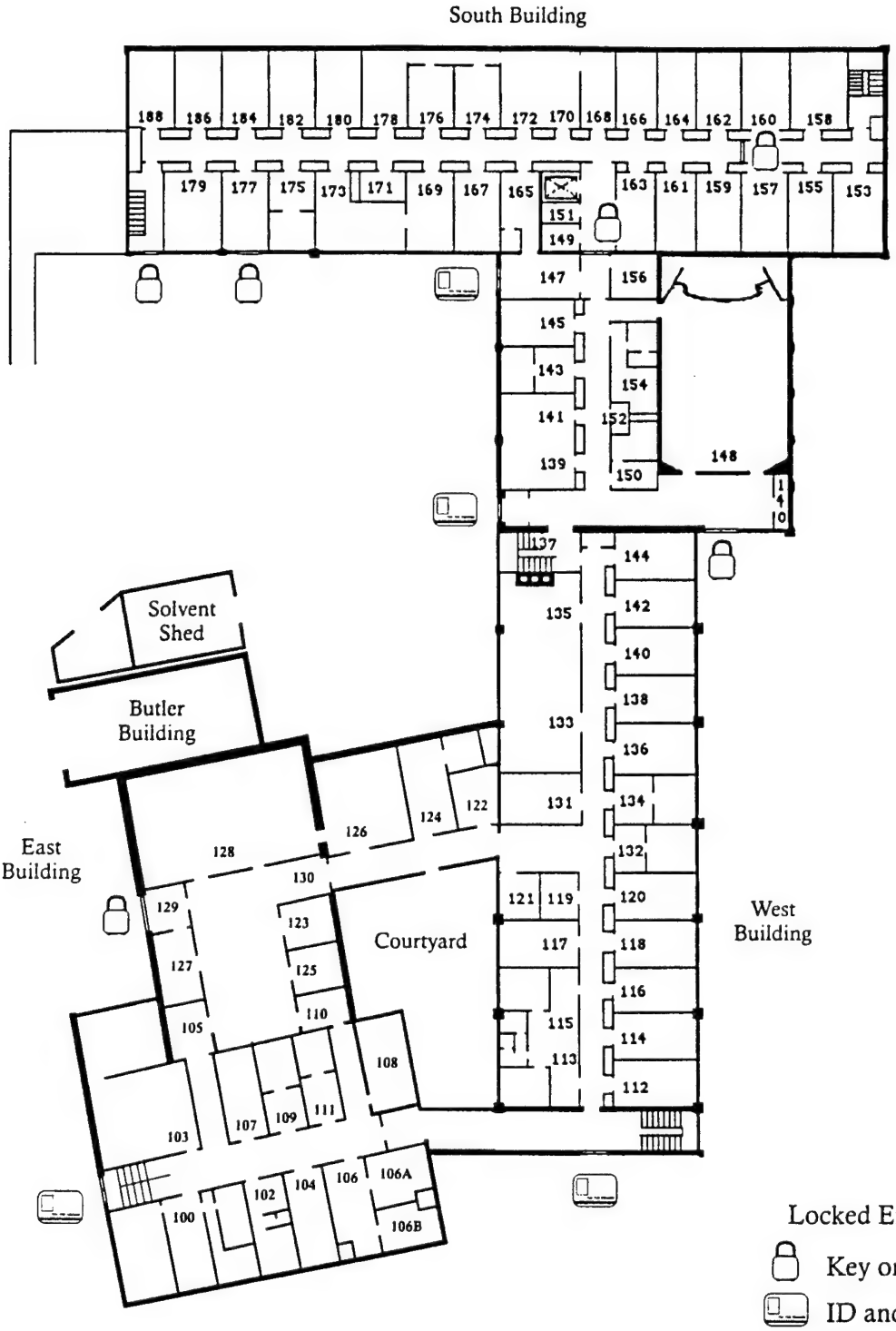
Figure 2


Effect of Pyridostigmine on Habituation of Rearing
PB mg/kg
0
10
30
Counts / 5 minutes
25
20
15
10
5
0
0
20
40
60
Minutes

2/01

First Floor
New York University

Figure 3.



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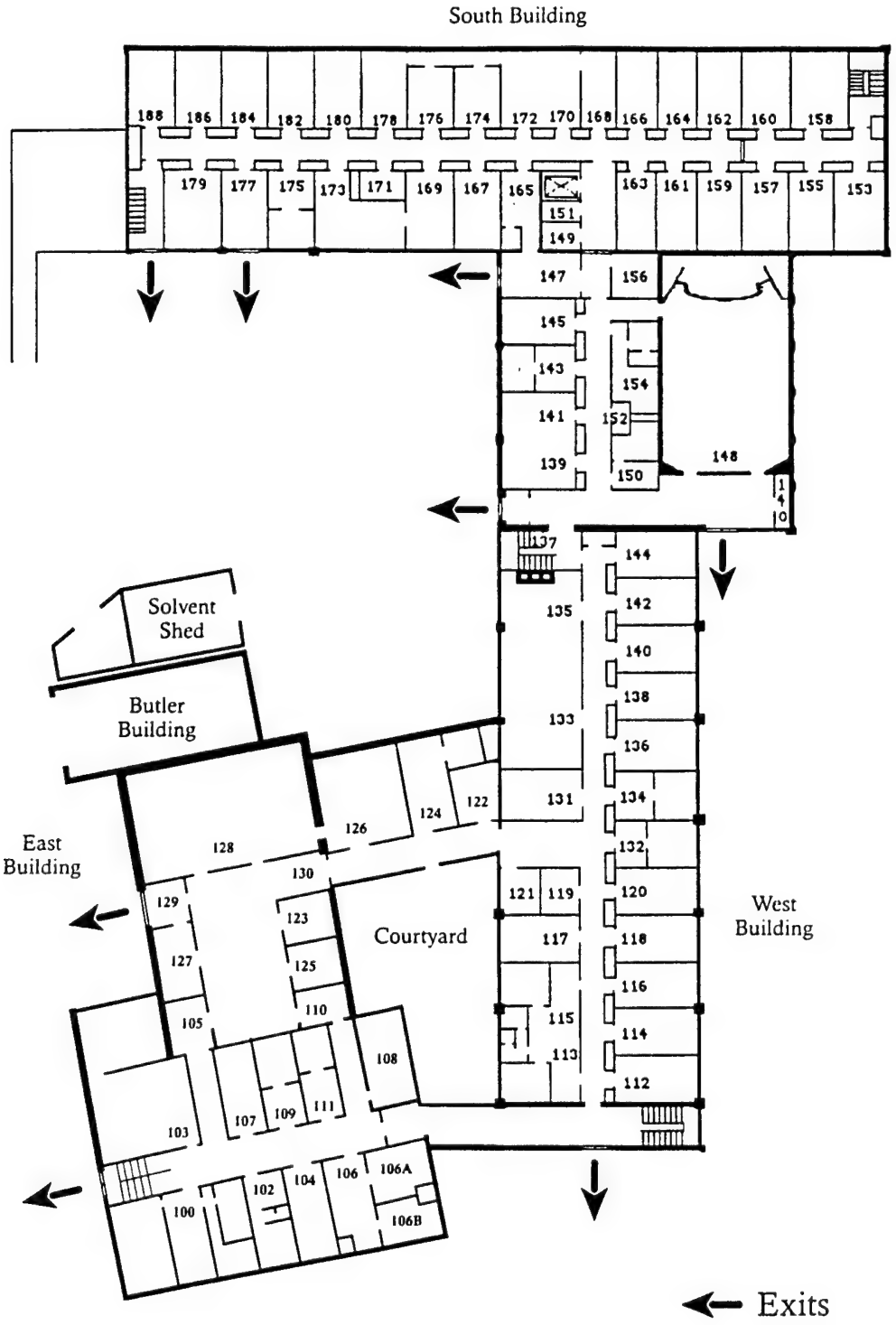
First Floor
New York University

Figure 4.



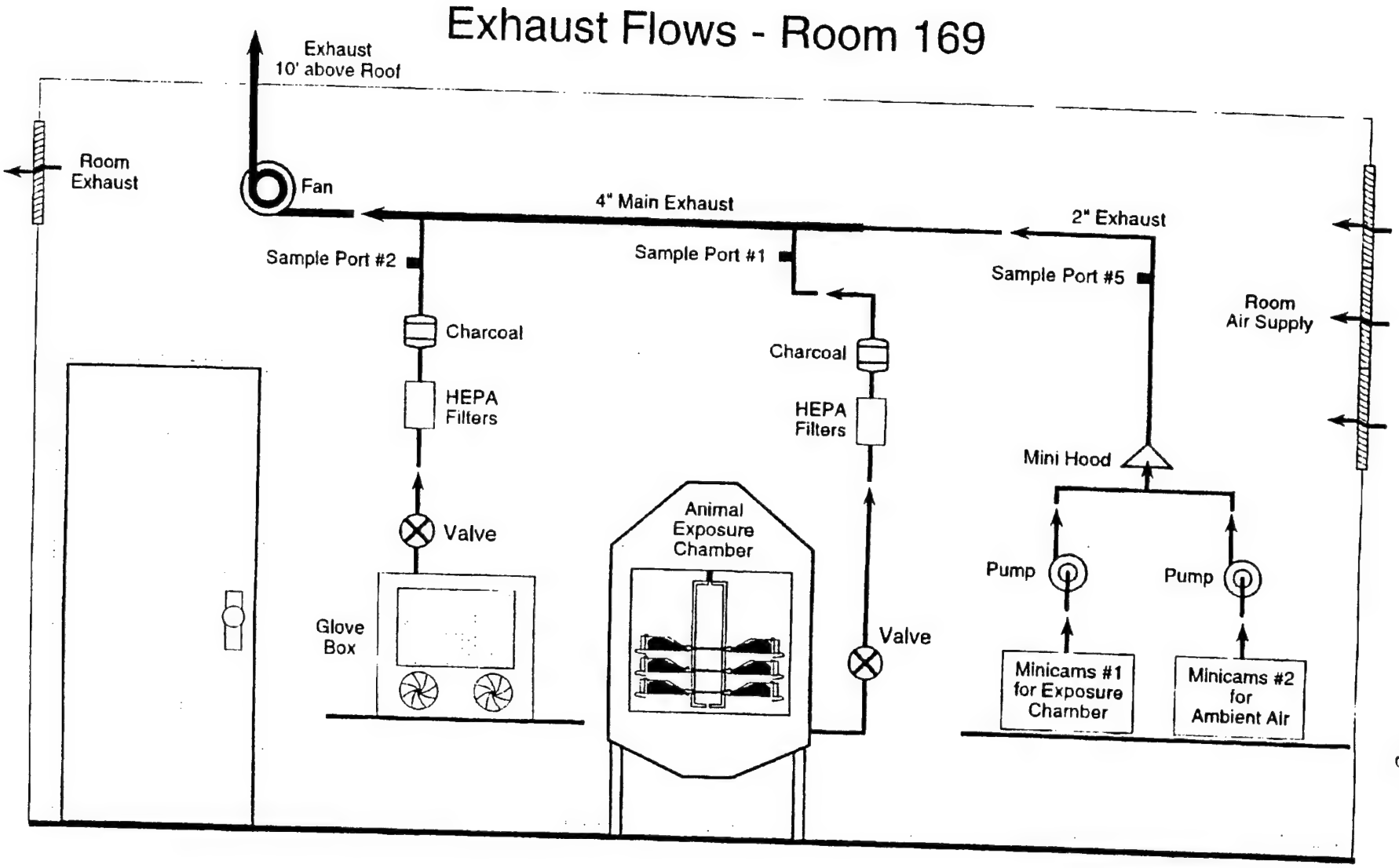


Figure 5.

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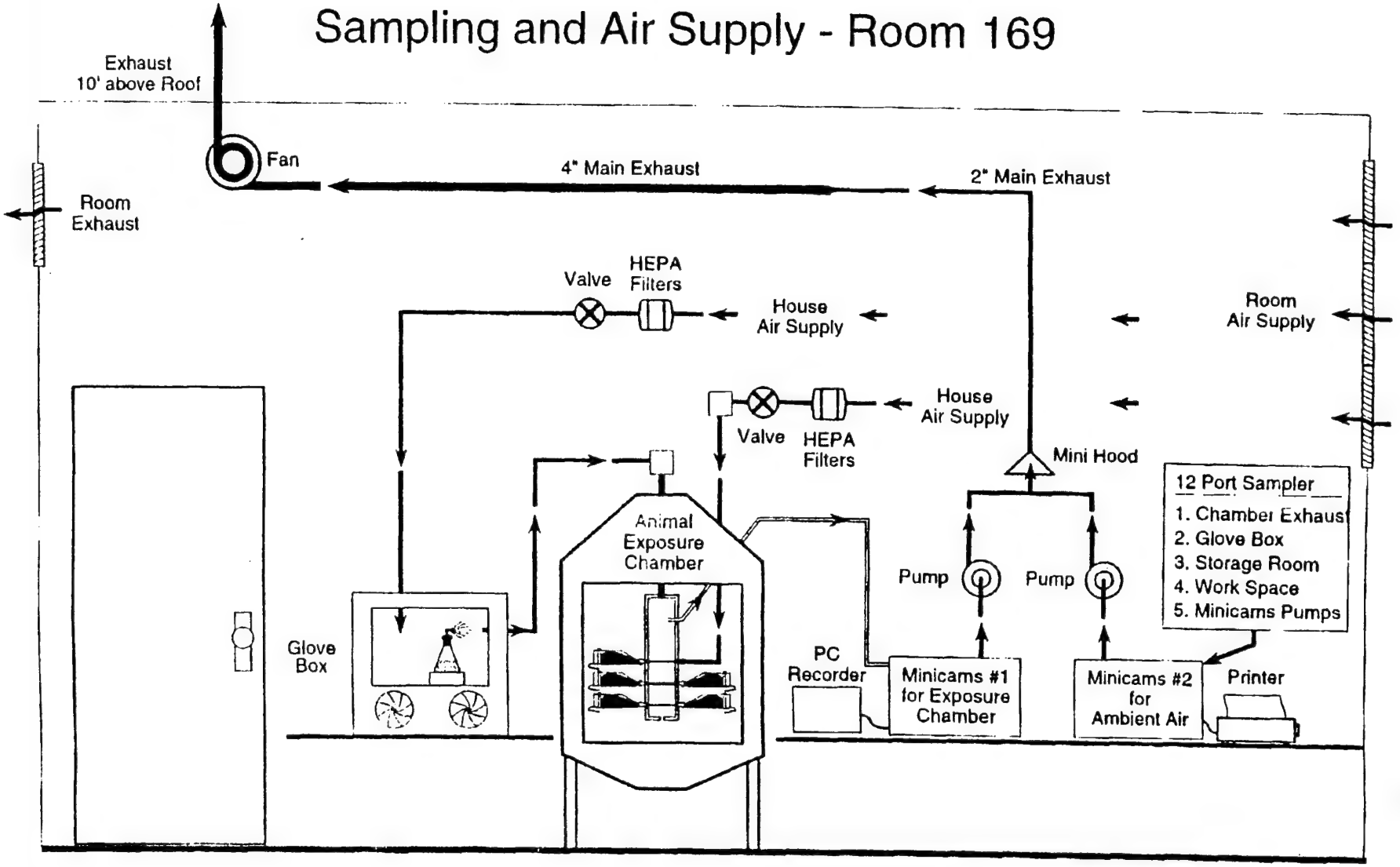
Sampling and Air Supply - Room 169
Exhaust
10' above Roof
Fan
4" Main Exhaust
2" Main Exhaust
Room
Exhaust
Valve
HEPA
Filters
House
Air Supply
Room
Air Supply
House
Air Supply
Valve
HEPA
Filters
Mini Hood
12 Port Sampler
1. Chamber Exhaust
2. Glove Box
3. Storage Room
4. Work Space
5. Minicams Pumps
Animal
Exposure
Chamber
Pump
Pump
Glove
Box
PC
Recorder
Minicams #1
for Exposure
Chamber
Minicams #2
for
Ambient Air
Printer

Figure 6.

FUNDAMENTAL AND APPLIED TOXICOLOGY 6, 721–732 (1986)

A System for Assessing Toxicity of Chemicals by Continuous Monitoring of Homecage Behaviors[1]

H. L. EVANS,[2] P. J. BUSHNELL,[3] J. D. TAYLOR, A. MONICO, J. J. TEAL,[4] AND M. J. PONTECORVO[5]

Institute of Environmental Medicine, New York University Medical Center, 550 First Avenue, New York, New York 10016

A System for Assessing Toxicity of Chemicals by Continuous Monitoring of Homecage Behaviors. EVANS, H. L., BUSHNELL, P. J., TAYLOR, J. D., MONICO, A., TEAL, J. J., AND PONTECORVO, M. J. (1986). *Fundam. Appl. Toxicol.* **6**, 721–732. A noninvasive system is described for continuous recording of behaviors in the home cages of rats. Commercially available mesh cages were used so as to conform with housing conditions in most toxicological studies. A minicomputer controlled environmental lighting and recorded eating, drinking, rearing, and horizontal activity. The system's sensitivity was comparable to more complex systems. Validity was demonstrated through manipulation of environmental lighting, food deprivation, and the effects of amphetamine, scopolamine, ethanol, methylscopolamine, triethyltin, and trimethyltin. Advantages over other systems are practicality, economy, the simultaneous analyses of several naturalistic behaviors of individual rats, and the quantification of diurnal rhythms.

Appetite for food and water, and the daily cycle of activity and quiescence are sensitive indicators of the effects of drug and diseases (Moore-Ede *et al.*, 1982; Reiter and MacPhail, 1982) including occupational exposure to metals or solvents (Smith *et al.*, 1970; Goldberg and Cranmer, 1986). These endpoints also are sensitive in experimental models of such exposure (Bushnell and Evans, 1985a,b; Dempster *et al.*, 1984; Berthoud *et al.*, 1976). Research methods used to study these endpoints (Terman *et al.*, 1984; Stoff *et al.*, 1983; West *et al.*, 1983), do not permit the continuous observation of a sufficient number of animals for the evaluation of toxicant-induced changes in diurnal rhythms. Reiter *et al.* (1978) measured the combined activity of groups of rats to document lead-induced changes in diurnal cycles; however, observation of individual animals provides greater statistical power with equal efficiency and less cost.

The number of animals needed for toxicity testing might be reduced by using methods permitting intensive study of several variables in each animal. Cage-side observation, common in screening for behavioral toxicity, has several disadvantages: (1) observations must be made in the light when rodents are normally inactive; (2) The observer's presence can alter on-going behavior; (3) most observations yield ordinal rather than continuous measures, limiting statistical power; and (4) interobserver reliability is difficult to establish.

We now describe and validate a practical system for continuous, automated measurement of four naturalistic behaviors (eating, drinking, rearing, and horizontal activity) for

[1] This work was supported in part by a contract (ES-2-5017), a Center Grant (ES-00260), and by a Training Grant (ES-07065), all from the National Institute of Environmental Health Sciences.

[2] To whom reprint requests should be addressed.

[3] Present address: Health Effects Research Laboratory, Neurotoxicology Division, MD-74B, U.S. Environmental Protection Agency, Research Triangle Park, N.C. 27711.

[4] Present address: Toxicology Department, Avon Products, Inc., Division St., Suffern, N.Y. 10901.

[5] Present address: Nova Pharmaceuticals, Inc., Baltimore, Md. 21224-2788.

0272-0590/86 $3.00

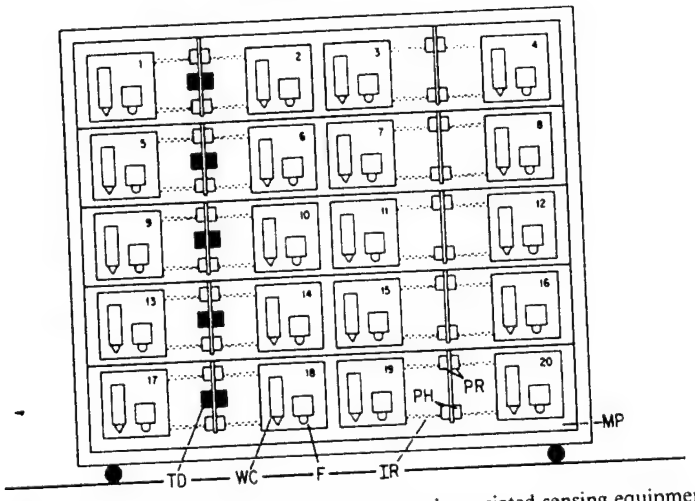

FIG. 1. Schematic arrangement of one rack with 20 cages and associated sensing equipment. Individual rat cages are numbered. For illustrative purposes, position of touch detectors (TD) used to sense eating and drinking is shown only on left side of rack. The position of photocells to sense rearing (PR) and horizontal activity (PH) are shown on both sides. Path of retroreflective photobeams is indicated by IR. MP = clear acrylic mounting panel to isolate water canister (WC) and feeder (F) from rack and cages.

each of 40 rats. Since the effects of toxicants on locomotor activity are dependent on the apparatus employed (Reiter and MacPhail, 1982; Tilson and Mitchell, 1984), the present system used the rat's living environment itself as the test apparatus. Nonintrusive measurements in the home cage avoid transferral to test apparatus or severe motivational manipulations (*e.g.* food deprivation or electric shock). Commercially available components were used to minimize the need for fabrication and to maximize uniformity. The hardware for sensing eating, drinking, rearing, and horizontal activity cost less than $400/rat.

EXPERIMENTAL SYSTEM

Racks of cages. We used stainless-steel, single-depth racks[6] containing 20 individually caged rats and 10 equipment clusters. Each rack had 5 rows with 4 rat cages/row and 2 equipment clusters/row (Fig. 1). Placement of equipment adjacent to the rat cage, rather than on or inside the cage, permitted independent access to the rat or equipment. Thus, equipment was serviced without directly contacting cages or rats, and removal of rats for weighing, dosing, and cage washing did not disturb the equipment mounted adjacent to each cage.

The rats were housed individually in suspended stainless-steel cages[7] with solid walls on the back and on one side, and 1.3-cm mesh on the front, bottom, and the second side. The cage dimensions (17.8 cm w × 30.0 cm l × 20.3 cm h) provide ample space for behavior. Unlike plastic "shoe box" type cages, hanging wire cages require less frequent cleaning, have no protrusions from the top to influence activity, and require no bedding which can block photobeams or introduce additional variability (Vesell *et al.*, 1976). Stainless steel also minimizes uncontrolled exposure to metals and provides a good reflective surface for the photocells (see Horizontal and vertical activity). Aluminum mounting brackets between cages supported photocells and acrylic brackets along the rear of the rack supported and electrically isolated the feeders, water canisters, and touch detectors (see below and Figs. 1 and 2).

Food consumption. Rats had *ad libitum* access to 45 mg food pellets.[8] Since one pellet approximates one "bite" of the rat's normal food intake (Panksepp, 1973), the pellets are not hoarded nor crumbled to the extent of conventional lab chow. A 50 g supply of pellets was contained in a 2-

[6] Wahmann No. LC-385/NS, Hazleton Systems, Inc. Aberdeen, Md. 21001.

[7] Wahmann No. LC-385/SA, Hazleton Systems, Inc.

[8] Precision food pellets, Bio-Serv Corp., Frenchtown, N.J.

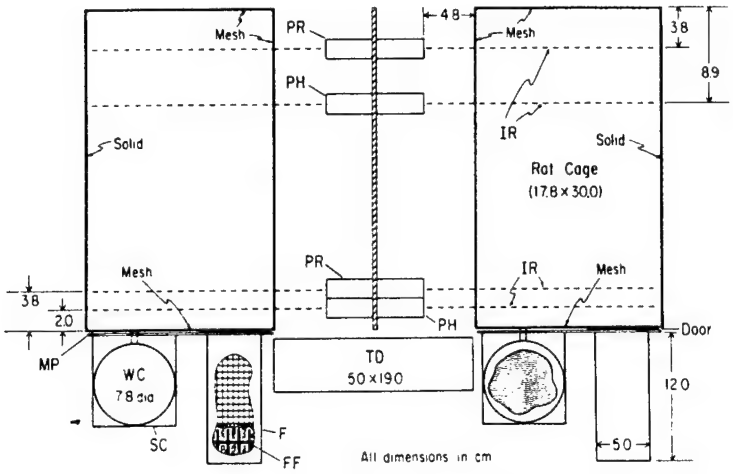

FIG. 2. Detailed diagram showing two cages and associated equipment, viewed from above. Distances shown in cm. SC = acrylic shelf holding WC. FF = Follower in Feeder. Door can be closed to bar entry to feeder. Other symbols as in Fig. 1. Rats cannot see other rats in adjoining cages.

part feeding chamber[9] located outside the cage. This chamber minimized hoarding, spilling, or contamination with urine and feces. A plastic drawer containing the food pellets was detached from the bottom of the chamber for weighing to determine the total food intake and thereby the total number of pellets eaten. The rat entered this feeder through an opening (6.3 cm w × 5.3 cm h) cut in the cage mesh (Fig. 2). The recessed feeder and positioning of the lower photobeam at this end of the cage eliminated artifactual activity counts from movements of the rat while eating, i.e., the beam was near the feeder entrance so that it was continuously interrupted while the rat was in the feeder. Rats were not required to work for food, as this could have altered the diurnal pattern of eating (Popplewell and Burton, 1984; Falk, 1971).

To permit automatic sensing of pellet removal, the feeder was modified by inserting a 16-gauge, stainless-steel rod vertically through a hole in the bottom of the plastic drawer and soldering to it a 5 × 3-cm piece of stainless-steel mesh (1.3 cm) which rested horizontally on top of the pellets. This mesh and rod was referred to as a "follower," since it followed the level of the pellets down as the rat removed them. The end of the rod protruding through the drawer was attached to a touch detector.[10] As the rat ate pellets, contacts with the follower were detected without current flowing through the rat. Photocell systems would have required greater precision in alignment.

Water consumption. A 500-ml disposable plastic pouch containing chlorinated spring water[11] was placed in a stainless-steel canister with a drinking spout. This system eliminates dripping, evaporation, breakage, cross-contamination, and bottle washing. Each water canister, mounted next to the feeder on an acrylic panel, was electrically isolated from the rack of cages (Figs. 1 and 2). The drinking spout, with a pressure-sensitive valve to prevent leakage, was located just outside the cage, 3.5 cm above the cage floor. The rat licked the spout through a hole (2.5 cm w × 3.5 cm h) cut in the cage mesh. This position minimized counts caused by non-licking behaviors.

Photocell devices for detecting licking were either too expensive or were too prone to misalignment. Weighing conventional water bottles overestimated fluid consumption due to leakage and evaporation (Table 1) and could not provide sufficient temporal precision. Commonly used lick-detecting circuits (e.g., West *et al.*, 1983) may interfere with taste by requiring current to flow through the rat (Martonyi and Valenstein, 1971) and because their circuitry must be completed with wires attached to the cage which hinder dosing and cage cleaning. Therefore, licking was detected with a touch detector[10] identical to that used to detect eating. These units, capable of registering input rates of 10/sec, were used successfully to measure licking by mice (Dempster *et al.*, 1984; Teal and Evans, 1982).

Body weight and other weighing operations. An electronic balance, with integrating circuit and digital display,[12] was used to weigh the rats, water canisters, and feeders

[9] Nalgene No. 6500104, Sybron Corp., Rochester, N.Y.

[10] Sobco capacitance-discharge, contact-sensing circuit, Cambridge, Mass.

[11] Cold Spring Products, Inc. Stanfordville, N.Y. 12581.

[12] Sartorius No. 1403-MPZ, Sybron/Brinkmann Co., Westbury, N.Y. 11590.

with an accuracy of ±0.1 g. These data were collected not more than once per 24 hr.

Horizontal and vertical activity. Photocells[13] employing infrared (ir) light-emitting diodes (LED) were used for sensing activity because ir light is invisible to rodents and because of the long life span of the LED. Photocells were mounted in clusters between the rat cages so that the light emitted from the LED passed through the near mesh wall of the cage and was reflected back from the far solid wall of the cage (Figs. 1 and 2). This retroreflective feature reduced the critical alignment required by most photocells, and conserved space by housing both the ir source and detector in a single unit.

Two of the photocells were mounted 3.8 cm below the cage top and 3.8 cm from the front and rear walls of the cage and their combined output was counted as vertical activity (rearing). Two additional photocells were mounted 3.8 cm above the cage floor and their combined output was counted as horizontal activity. The low photocell near the feeder was 2.0 cm from the cage wall; the other low photocell was mounted 8.9 cm from the rear wall of the cage (Fig. 2).

Data acquisition. Control of the room lighting and the acquisition of data was performed by a minicomputer[14] and interface.[15] This system provided a proven high-level language with sufficient temporal precision (0.01 sec) and memory to manage data from at least 50 rats (200 input lines). Data were periodically transferred to the system's magnetic disk for permanent storage, for additional processing, and for transfer to a larger system for statistical analyses and graphics.

PROCEDURES AND RESULTS

Animals

All animals were male Fischer-344 rats.[16] weighing 150 g upon arrival in the laboratory at 8 weeks of age. All rats were housed individually[6,7] in a room maintained at 22.2 ± 1°C with relative humidity of 45–70% and diurnal lighting (less than 5.0 foot-candles at the level of the cages) provided by overhead fluorescent tubes. Nocturnal illumination was provided by six 25-W red incadescent bulbs. Animal care conformed to NIH guidelines (1980).

Growth of Rats Maintained on the System

To determine whether the new system influenced the growth of normal rats, 20 rats were housed in the new system and their body weights were compared with 9 conventionally housed rats in the same room over an 80-day period. Conventionally housed rats had *ad libitum* access to tap water in glass bottles with stainless-steel sipping tubes, and to food[17] in a mesh feeder suspended on the exterior of the cage. Conventionally housed rats had neither photocells nor touch sensors attached to their cages. The two housing conditions resulted in identical growth from an initial mean of 160 g to a final mean of 180 g, in good agreement with other data (Witman *et al.*, 1984). Therefore, the new system did not modify growth, health, and maturation.

Quantification of Food and Water Consumption

The water canister and feeder were weighed daily to determine food and water consumption. Daily food consumption varied with the age and size of the rats. Young rats weighing an average of 190.4 g ate 14.4 g food, while the same rats, when older, weighed 283.0 g and ate 17.9 g food. Under normal conditions, 50% of the variance in daily food consumption was accounted for by the previous day's metabolic mass (body weight$^{0.75}$). A mean of 32 mg food was consumed per touch of the follower. This statistic provides an index of feeding efficiency with potential utility for the detection of neurotoxicity.

Water consumption varied less with age than did food consumption. Young rats mentioned above drank 16.2 g water daily; they drank 16.8 g when older. The volume of water consumed per lick (drinking efficiency) averaged 3.2 μl (SD = 0.5 μl), a statistic analogous to feeding efficiency.

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[13] No. A1100, Autotron Inc., Danville, Ill.

[14] No. PDP8A, Digital Equipment Corp., Maynard, Mass.

[15] SUPERSKED, State Systems Inc., Kalamazoo, Mich.

[16] Harlan Industries, Indianapolis, Ind.

[17] Rodent Lab Chow, Ralston Purina Corp., St. Louis, Mo.

For comparison of water consumption with the new and conventional systems, water bottles of conventionally housed rats were weighed at the same time that water canisters from the new system were weighed. Canisters and bottles from unoccupied cages also were weighed daily. Substantial variability and overestimation of water consumption, based upon the weight loss of conventional water bottles (Table 1) was apparently due to dripping, evaporation, and spillage during weighing, and non-drinking contacts with the drinking spout by the rats. This extraneous water loss would distort estimates of exposure to toxicants via the drinking water. In comparison, estimates of water consumption from canisters contained negligible loss of water from such extraneous factors.

Coefficients of Variation

To evaluate the variability inherent in the dependent variables, we calculated coefficients of variation for the endpoints employed by this system (Table 2). The between-rat coefficient describes the variability among 19 different rats on a typical day without exposure to toxicants. The second coefficient describes within-rat variation across four consecutive days of observation as the mean coefficient from 19 individual rats.

TABLE 1

DAILY DECLINE IN WEIGHT OF WATER CONTAINER: INFLUENCE OF TYPE OF CONTAINER, HANDLING, AND DRINKING

	Canister		Stoppered bottle	
	One rat[a]	No rat[b]	One rat[a]	No rat[b]
	Change in weight (g per 24 hr)			
Mean	19.9	0.4	59.6	5.0
SE	1.1	0.1	3.0	0.4
N	10	5	10	3

[a] Cage, as described in text, with one rat (290 g average body weight).

[b] No rat = empty cage with water container removed for weighing and, in case of canister, valve check, every 24 hr.

TABLE 2

COEFFICIENTS OF VARIATION[a]

	Water			Food		Activity	
	Can (ml)	Bottle (ml)	Licks	g	Bites	Horiz	Vert
Between rats	0.17	[b]	0.31	0.11	0.35	0.27	0.67
Within rats	0.11	0.15	0.14	0.10	0.19	0.51	0.40

[a] Coefficient of variation = $SD/\bar{X}$. N = 19 under non-drug conditions.

[b] Not determined.

Entrainment of Feeding Behavior

A useful system should be able to document diurnal cycles of behavior which are closely controlled by the environmental lighting. To document the diurnal pattern, homecage behaviors were recorded at 2-hr intervals around the clock. Both room lighting and the 2-hr data collections were controlled by the computer, thus ensuring temporal precision.

The entrainment of food intake by the environmental lighting is demonstrated in Fig. 3. After having been maintained by the animal supplier[14] on a regular cycle of 12-hr light and 12-hr dark, the rats were moved to the present laboratory and the LD cycle was also advanced by 4 hr. With reference to Fig. 3, rats accustomed to darkness from hours 4 to 16 were now exposed to darkness from hours 0 to 12. People worked in the room only during the last hour of the light period (hours 23 to 24, corresponding to 2–3 PM local time). After 6 days under the new LD cycle (Fig. 3, upper panel), the rats still ate mostly at the time of day corresponding to the previous dark period (4 to 16 hr). After 16 days under the new LD cycle (Fig. 3, middle panel), the period of greatest eating had shifted to the start of the new dark period (0 to 2 hr) with very little eating during the light period (hours 12 to 24). However, a substantial amount of eating remained at hours 10 to 12. After 16 days, entrainment was nearly complete, as shown by

the similarity of the diurnal patterns on Days 16 and 77. Day 77 provides a reference point for complete entrainment (Fig. 3, lower panel).

Effects of Food Deprivation

Food deprivation induces well-known changes in water consumption and locomotor activity of rodents, which a new system should be able to document. After 13 weeks of *ad libitum* feeding, rats were deprived of food by closing the feeder door (Fig. 2) for 24 hr starting with light offset. Activity and water consumption were monitored before, during, and for 2 days after food deprivation. Mean body weights fell from 297 to 279 g after 24-hr deprivation. Weights returned to 296 g within 24 hr after access to food was restored.

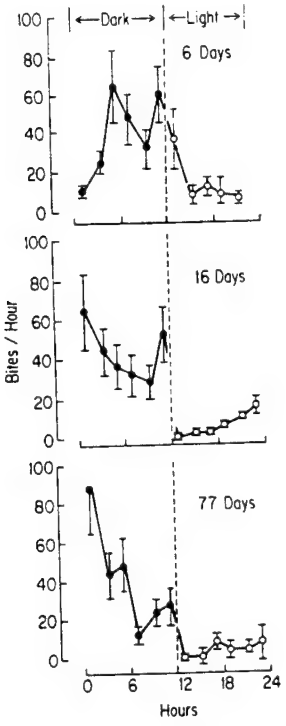

FIG. 3. The entrainment of the diurnal feeding cycle after a 4-hr shift in the LD cycle. Data reflect the mean ± 1 SE (N = 20) number of touches on the followers, determined at 2-hr epochs during the dark, (filled circle) and during the light (open circle). Mean body weight was 190 g when LD cycle was shifted. Entrainment progressed from the 6th day after the shift in the LD cycle (top panel), to the 16th day (middle). Entrainment was nearly complete by the 16th day, since the diurnal pattern was similar to the pattern on the 77th day (bottom) long after entrainment was complete.

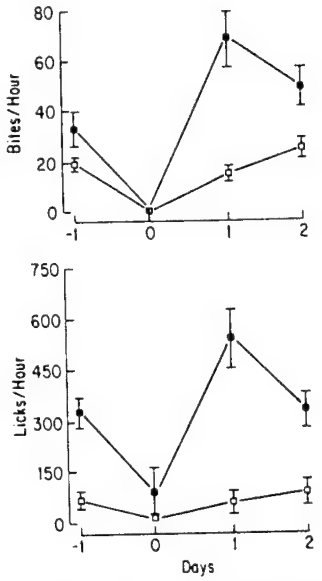

FIG. 4. The effects of food deprivation on eating (upper) and drinking (lower). Nocturnal ingestion (filled points) and ingestion in the light (open points) are shown separately for 12-hr periods. Brackets indicate where ±1 SE exceeds size of symbol (N = 20). The baseline data (*ad libitum* access to food) is shown as Day −1 and the behavior during the 24 hr without access to food is Day 0. Behavior during the first and second day of return to food is shown as Day 1 and Day 2, respectively. Note that no feeder counts occurred when access to the feeder was barred (Day 0, upper panel). Water consumption was markedly reduced during this food deprivation (Day 0, lower panel). Mean weight was 297 g on Day −1.

Food deprivation caused the expected decline in drinking, with a rebound in both drinking and eating on the first day of renewed access to food (Fig. 4). Deprivation also increased nocturnal activity, but had no significant effect upon activity during the light (Fig. 5). Nocturnal vertical activity remained elevated longer than nocturnal horizontal activity after food deprivation (Fig. 4), suggesting that it might provide more lasting evidence than horizontal activity of physiological challenge. Both measures of ingestive behavior (Fig. 4)

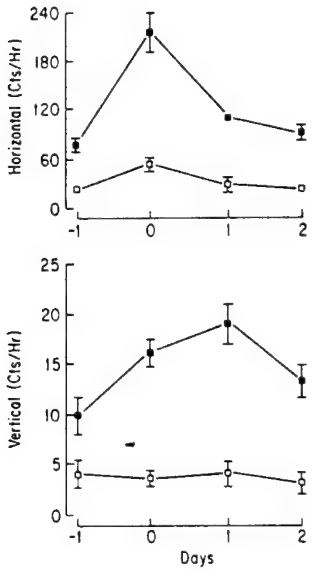

FIG. 5. Effect of food deprivation on horizontal (upper panel) and vertical (lower panel) activity. Rats and symbols are the same as in Fig. 4. Note that activity increased during deprivation and recovered with 2 days.

and of activity (Fig. 5) indicated recovery on the second day after reaccess to food.

Drug Effects

To manipulate homecage behaviors pharmacologically, amphetamine and scopolamine were injected ip 5 to 20 min before light offset, and data were collected for the first hour of the dark period. Each rat received vehicle and each drug dose according to a randomized repeated treatments design on Tuesdays and Thursdays. (−)-Scopolamine hydrobromide[18] (0.2, 0.6 and 1.8 mg/kg) or (−)-methylscopolamine bromide[18] (1.8 mg/kg) were administered to one group of rats in a constant volume of 1.0 ml/kg and *d*-amphetamine sulfate[19] (0.3, 1.0, and 3.0 mg/kg) was administered to a different group at 0.5 ml/kg.

Both drugs markedly increased horizontal and vertical activity during the first hour after injection (Fig. 6). However, scopolamine was much more potent in increasing activity than was amphetamine. As expected, methylscopolamine, at a dose equal to an effective dose of scopolamine, had little more effect than did sham injection.

Moderate doses of amphetamine severely reduced both eating and drinking in the hour following injection (Fig. 7). However, amphetamine did not significantly alter the more common toxicological endpoints, i.e., body weight or total water and food consumption in the 24 hr following injection. These results indicate that the greater temporal resolution afforded by the present system may facilitate detection of short-term responses missed by conventional toxicity tests.

In contrast to amphetamine, scopolamine severely reduced eating (Fig 7, top) but not drinking (Fig. 7, bottom). Instead, drinking was slightly elevated following scopolamine. Methylscopolamine and scopolamine were equally effective in altering eating and drink-

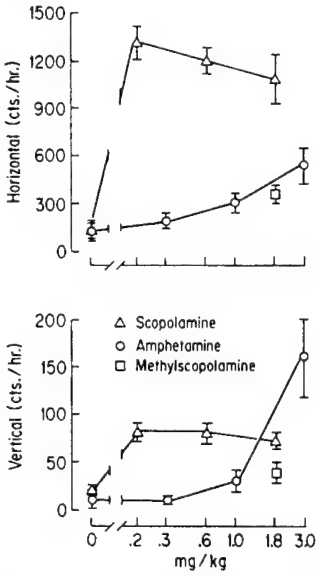

FIG. 6. Effects of *d*-amphetamine (circle), (−)-scopolamine (triangle), and (−)-methylscopolamine (square) upon nocturnal activity recorded for 1 hr starting 5 to 20 min after ip injection. The zero dose was physiological saline. *X* axes are log scale. All doses were calculated as the salt. Points represent the mean ± SE (N = 20).

[18] Sigma Chemical Co., St. Louis, Mo.
[19] Pennwalt Corp., Rochester, N.Y.

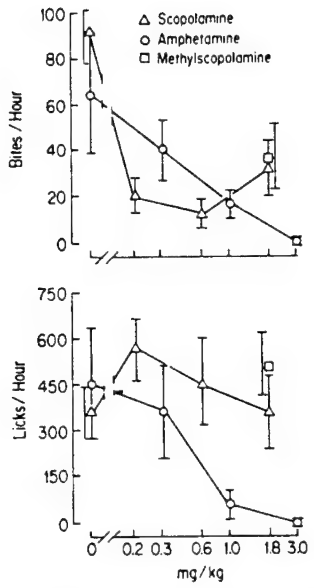

FIG. 7. The effects of amphetamine, scopolamine, and methylscopolamine upon eating (upper panel) and drinking (lower panel). Rats and data format are the same as in Fig. 6. Baselines differed in the scopolamine and amphetamine experiments (0 mg/kg, upper panel) because of growth in the time between the two experiments.

ing, in contrast with their differential effects on activity (Fig. 6).

Effects of Alkyltins

The system's capability for continuous recording was used to demonstrate a gradually emerging neurotoxicity from trimethyltin chloride[20] (TMT) and triethyltin bromide[21] (TET). Homecage behaviors were recorded continuously over 5 consecutive days after oral exposure to either TMT (n = 5) or TET (n = 10) or vehicle. Figure 8 shows the diurnal rhythm in vertical activity, an endpoint that was sensitive to both of these alkyltins (Bushnell and Evans, 1985a,b).

All rats exhibited much more activity in the dark than in the light, indicating that the rats remained sensitive to environmental lighting after exposure to TMT or TET. Vehicle-treated rats exhibited a pattern of activity reported by Mondon *et al.* (1985): one distinct burst of activity at the start of the nocturnal period and a second near the end of the nocturnal period. Both 7 mg/kg TMT (Fig. 8, lower panel) and 3 mg/kg TET (upper panel) caused a decline in the high levels of vertical activity at the end of each nocturnal period (last point in "Dark" in Fig. 8). Furthermore, TMT disrupted the diurnal rhythm so as to produce significant hyperactivity in the light period on Day 3, indicating the advantage of continuous observation: shorter observation periods could have produced seemingly contradictory results, depending on the time of measurement.

Figure 8 also indicates the system's sensitivity to ethanol which served as a vehicle for TET. The effect is evident in the comparison of the two vehicle groups on Day 1: 0.4 g/kg ethanol was administered to the vehicle group in the upper panel and water to the vehicle group in the lower panel. Ethanol significantly suppressed vertical activity 12 hr after dosing, compared to water vehicle. Recovery from ethanol is indicated by the similar diurnal patterns of the two vehicle groups on Day 2.

DISCUSSION

A system for continuous recording of four naturalistic behaviors of 40 rats in commercially available homecages has proven promising for toxicity testing. The most comprehensive previously reported system monitored 16 rats in non-standard cages (Stoff *et al.*, 1983), and other systems have employed from two to five rats (Evans, 1971; West *et al.*, 1983; Terman *et al.*, 1984). By monitoring rats individually, rather than in groups, the present system provides a larger N for statistical power and better identification of individuals who may be outliers.

A minicomputer controlled environmental lighting and monitored diurnal patterns of

[20] ICN Biomedicals, K & K Labs, Plainview, N.Y.
[21] Alfa Products, Danvers, Mass.

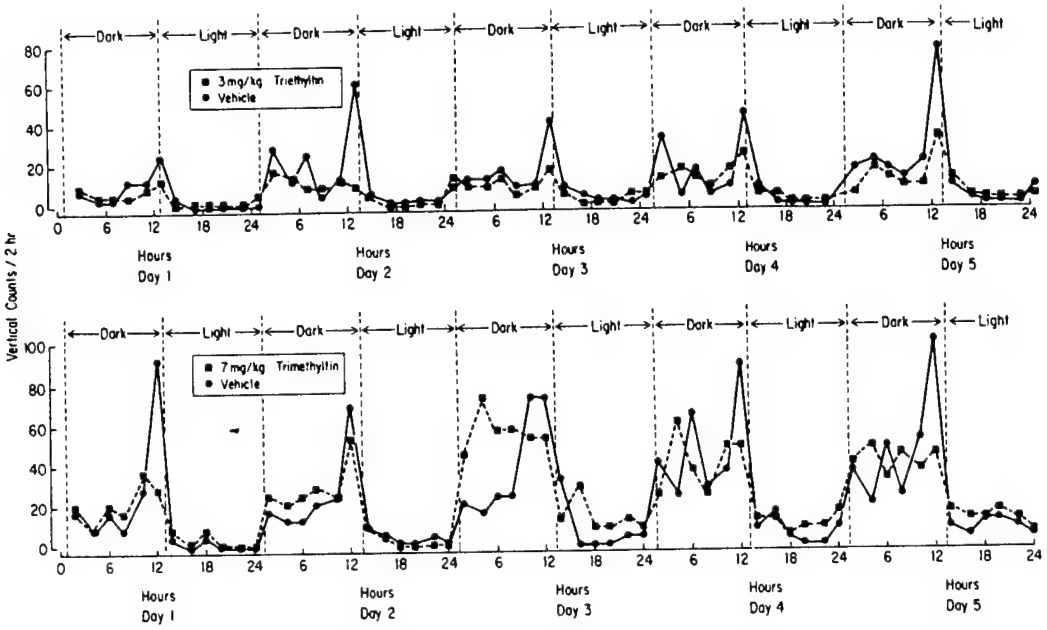

FIG. 8. Continuously recorded diurnal patterns in vertical activity (rearing). Upper panel: effect of acute exposure to 3 mg/kg TET or vehicle (15% ethanol in water). Lower panel: effect of 7 mg/kg TMT or vehicle (distilled water). Data recorded continuously in 2-hr epochs starting immediately after oral intubation (hour 0, Day 1). On certain days data collection was interrupted from 2300 to 2400 hr when rats were weighed and feeders were refilled. This occurred on Day 2 in upper panel and on Days 2 and 4 in lower panel. No other handling nor human activity occurred in the room at other times. Rats receiving TET (N = 10 per treatment group) were slightly older (body weight ranged from 310 to 358 g) than were rats receiving TMT (N = 5 per treatment group, body weight range from 266 to 316 g).

eating, drinking, rearing, and horizontal activity. The baseline variability of these endpoints (Table 2) is within the range of other physiological and behavioral measures (Kissileff and Van Itallie, 1982; Dews, 1982). Since these estimates of variability include both experimental error and normal biological variation, the system's remote sensing of behavior and automated data collection should minimize the former and permit the latter to indicate toxic effects. The sensing equipment is economical and the system is not labor intensive. The most frequent service requirement is refilling of the feeder (a 200 g rat empties its feeder every 3 days). Rats grow normally and can be maintained indefinitely in the system.

The system's validity was demonstrated by the entrainment of eating by environmental lighting (Fig. 3). Lighting is a major determinant of the temporal pattern of eating, drinking, and other activities of rodents (Moore-Ede *et al.*, 1982). Our results confirm that the rat's maximum eating occurs in the first hours of darkness (Terman *et al.*, 1984). Validity was further demonstrated by repeatable diurnal rhythms in activity (Fig. 8) and by the effects of food deprivation in reduced drinking (Fig. 4) and increasing nocturnal activity (Fig. 5). These consequences of a single day without food confirm classic findings (Verplanck and Hayes, 1953; Calvin and Behan, 1954). The results further indicate that the most significant changes occur during the nocturnal period; nocturnal behavior is often neglected in toxicological research because of the greater convenience of working in the lighted environment.

The system is sensitive to drugs and toxicants at doses of *d*-amphetamine or scopolamine equivalent to those reported to affect activity (Tyler and Tessel, 1979; Stoff *et al.*, 1983), eating (MacPhail and Gollub, 1974; Van Rossum and Simons, 1969), drinking (Evans and Patton, 1970), and operant behavior (Evans *et al.*, 1973; Dews and Wenger, 1977; Spencer *et al.*, 1985). Nocturnal rearing was significantly suppressed by 0.4 g/kg ethanol, a low dose for the rat (Mello, 1985). Another system did not detect effects of ethanol below 0.9 g/kg (Kulig *et al.*, 1985). Small and brief drug-induced changes (Figs. 6 and 7) could not be detected by conventional indices such as 24-hr food and water consumption. The system documented significant effects of TMT at 3 mg/kg (Bushnell and Evans, 1985a,b), indicating greater sensitivity than reported by others with behavioral (Ruppert *et al.*, 1982), neurochemical (O'Callaghan and Miller, 1984), or neuropathological endpoints (Chang and Dyer, 1985). The reduction in activity following 3 mg/kg TET (Fig. 8) is also seen following 1.5 mg/kg TET which is the lowest single dose of TET reported to affect the rat (Bushnell and Evans, submitted for publication).

As further evidence of validity, the system differentiated between peripheral antimuscarinic activity (methylscopolamine) and combined central and peripheral activity (scopolamine). Methylscopolamine and scopolamine were equally effective in reducing eating (Fig. 7), which was presumably due to reduced salivation; however, methylscopolamine was ineffective in increasing activity (Fig. 6), which presumably required CNS stimulation. The acute drug data demonstrate the system's advantage in providing simultaneous evaluation of multiple endpoints.

The method of water presentation minimized variability due to spillage and other errors (Table 1). Our index of drinking efficiency (3.2 μl/lick) is comparable to previous calculations ranging from 2.1 to 5.0 μl/lick (West *et al.*, 1983; Stellar and Hill, 1952). A comparable index of feeding efficiency proved sensitive to TMT (Bushnell and Evans, 1985a).

There are very few animal experiments on diurnal rhythms in toxicology. Changes in diurnal patterns have been induced by exposure to lead (Reiter *et al.*, 1978) or to TMT (Wenger *et al.*, 1984; Bushnell and Evans, 1985a,b). Continuous recording of vertical activity (Fig. 8) indicated both an increase and a depression of nocturnal rearing by TMT; this is an advantage of continuous recording over conventional, brief tests which had indicated only hyperactivity (Ruppert *et al.*, 1982; Swartzwelder *et al.*, 1981). Furthermore, continuous recording avoids the disruptive effects of handling when animals are transferred between home cage and test apparatus (Reiter *et al.*, 1978).

TMT clearly altered the diurnal pattern of activity without significantly affecting the 24-hr, total output of activity (Fig. 8; Bushnell and Evans 1985a). Thus the conclusion of Wenger *et al.* (1984) that the diurnal pattern is no more sensitive to TMT than the 24-hr total can be attributed to their use of less temporal precision (6-hr epochs) than ours (2-hr epochs). These findings suggest that the temporal pattern of homecage behavior provides one of the most sensitive and complete indices of toxicity. The present system provides a powerful tool for further investigations in this often neglected area of toxicology.

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Behavioral Changes in the Rat after Low Doses of Cholinesterase Inhibitors

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Behavioral Changes in the Rat after Low Doses of Cholinesterase Inhibitors. WOLTHUIS, O. L., AND VANWERSCH, R. A. P. (1984). *Fundam. Appl. Toxicol.* 4, S195–S208. In rats the acute effects of low doses of five cholinesterase (ChE) inhibitors were investigated in six behavioral tests. Considerable differences were found between the inhibitors studied. TEPP and sarin at doses up to 30% LD50 were without effects. In contrast, soman, physostigmine, and pyridostigmine caused effects at dose levels which did not produce overt symptoms and did not affect running speed and simple coordinated locomotion. Soman (≤3% LD50), physostigmine (≤4.5% LD50), and pyridostigmine (≤10% LD50) interfered with two-way shuttlebox avoidance learning, open field behavior, and complex coordinated movements (hurdle-stepping task). Tests of retention in a passive avoidance learning test appeared less sensitive. It is concluded that paradigms that involve higher CNS structures and require motor activity are sensitive to some ChE inhibitors at doses far below those that cause overt symptoms. The individual characteristics of ChE inhibitors play an important role. In contrast to TEPP and sarin, soman has a predominantly central effect. Further, the finding that pyridostigmine was also effective at unexpectedly low dose levels suggests that this compound may have more central actions than hitherto accepted.

Research on the behavioral effects of cholinesterase (ChE) inhibitors in the past has been mainly focused on memory effects (Deutsch, 1973; Moss and Deutsch, 1975) and on the development of tolerance upon repeated administration of inhibitors (Bignami *et al.*, 1975; Bignami, 1979; Giardini *et al.*, 1981; Russell *et al.*, 1981; Costa *et al.*, 1982; Costa and Murphy, 1982; Loullis *et al.*, 1983). Little work has been done on the acute behavioral effects of low dose levels that produce no overt somatic signs of intoxication, neither on the type of behavior that is most sensitive to disruption. Usually, the effects of a single compound—in many cases physostigmine—have been investigated in one or two behavioral tests (Vaillant, 1967; Rosecrans *et al.*, 1968; Kosić and Bignami, 1970; Holtzman and Jewett, 1971). Rarely has another carbamate (Goldberg *et al.*, 1965; Kurtz, 1977) or an organophosphate (Giardini *et al.*, 1981) been used.

Little distinction has been made between the different characteristics of the inhibitors. In particular, it has not always been clear whether the compound tested had a preferential peripheral action or had a predominant effect on the central nervous system (CNS). Such differences, however, might be crucial for the appearance of behavioral effects, especially at low dose levels. For example, Mertens *et al.* (1974) found in gerbils that the insecticide Phosdrin (mevinphos) started to induce behavioral decrements at dose levels that also caused overt signs of poisoning. In this case, the concomitant appearance of behavioral and somatic effects may have been caused by a purely peripheral (motor, sensory) action of the compound, since Sharma *et al.* (1973) reported that high Phosdrin doses which caused profound ChE inhibition in peripheral tissues of the rat had no effect on brain ChE activity. The outcome might have been different if an organophosphate (OP) with a preferential effect on the CNS had been tested.

In humans, no mental effects were noted in the absence of physical signs in a survey of 68 exposed insecticide workers plus 187 instances of suspected OP poisoning in other workers (Durham *et al.*, 1965). Unfortunately,

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the time interval between exposure and testing in this report was not specified. Most cases were probably chronically exposed and behavioral tolerance may have set in, Moreover, the large differences in the results obtained with the tests on mental deterioration may well have been due to the different characteristics of the OP's involved. Since the authors do not reveal the nature of the OP's to which the subjects had been exposed, the OP's may have been a mixed group of compounds which acted either predominantly peripheral or central. Nevertheless, these authors state that "mental effects generally have been more notable with nerve gases than with insecticides," without presenting much evidence for this statement.

In the present study the effects of five ChE inhibitors were compared in six different behavioral tests. The doses used caused no overt symptoms. It was found that two inhibitors had no effects in doses up to 30% LD50 in any of the tests, whereas three others caused behavioral effects in remarkable low doses. These differences were explained by the predominantly peripheral and central activity of these inhibitors, respectively. By comparing the minimal effective doses, it appeared possible to get an indication of which type of behavior might be particularly sensitive to the effects of centrally active ChE inhibitors. It was concluded that tests requiring both motor activity and the functional integrity of higher CNS centers were most sensitive.

If applicable to humans, the results might help to explain the relatively high incidence of "pilot error" accidents associated with depressed ChE activity in agricultural pilots (Smith *et al.*, 1968; see also Wood *et al.*, 1971). During military operations such effects might have serious consequences for pilots and personnel operating complicated equipment.

METHODS

Animals

Male small Wistar (WAG/Rij) rats with a body weight of 170 to 220 g and an age of approximately 3½ months were used in all experiments. The rats were bred in the laboratory under SPF conditions, i.e., hysterectomy derived, reared behind a germ protective barrier and bacteriologically controlled (for details see Baker *et al.*, 1979).

Toxicity Data

With few exceptions the doses used have been expressed as percentages of the LD50 values of the various compounds. These values were calculated according to Litchfield Wilcoxon from experiments with 6 groups of 10 rats each.

Apparatus and Procedure

Racetrack. In a 2-m Plexiglas alley with an electrifiable floor between startblox and goal platform, rats were trained until their photocell-recorded run times were minimal and stable. With three runs per day this usually took 3 days. On the fourth day, one of two tests were performed: either the animal made single runs 60 min before, just before, and 30, 60, and 90 min after the injection of the ChE inhibitor, or the animal made single runs 60 min before and just before the injection, whereas 30 min after injection five consecutive runs were made with the shortest possible time interval. Test 2 was intended as a "fatigue test."

Walking test (see Fig. 1A). The soles of the left fore and hindpaws of rats were dyed red and green, respectively. Subsequently, the animal was placed in a hollow vertically mounted Plexiglas wheel with a diameter of 50 cm and a inner width of 14 cm, rotating at a circumference speed of 14 cm/sec. A color TV camera, mounted under the wheel, registered the movements of the red and green footsoles through the transparent tread of the wheel on which the rat was walking. By automated electronic analysis and computer processing of the X and Y coordinates of the movements of red and green dots, parameters like step size, step duration, forward and lateral component of each step, coordination between fore- and hindpaw movements, etc., were objectively measured and analyzed. Of each rat 40 to 50 steps were analyzed, 30 min after the injection of a compound. A more detailed description of the method will be published elsewhere.

Hurdle-stepping test (see Fig. 1B). In essence, the device employed also consisted of a hollow wheel. The tread of the wheel consisted of four parallel stainless-steel grid rod hoops. The wheel had an inner diameter of 32 cm and a width of 8 cm. On the hoops 3-cm high and 1-cm thick hurdles (rungs) were mounted in a radial fashion and at equal distances. With a circumference speed of 5.4 cm/sec at the top of the rungs rats were trained for 30 min/day during 3 days to step from one rung to another. On the fourth day, the left hindpaw of the animal was dyed red and three 90-sec tests were carried out. The first test was a preinjection test, the second was performed 30 min

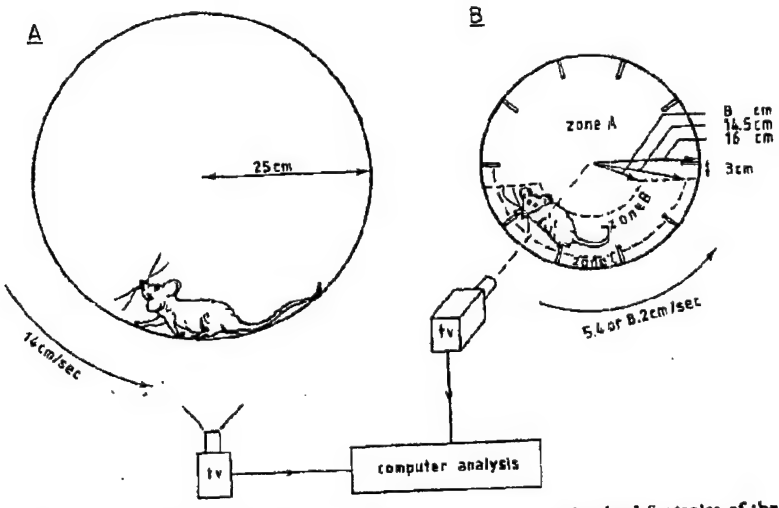


FIG. 1. (A) Walking test: the color TV camera was aimed upward at the dyed footsoles of the walking animal and registered at 50-Hz X and Y coordinates of the moving paws. (B) Hurdle-stepping test: an axially mounted color TV camera obtained a lateral view of the walking rat and registered the X and Y coordinates of the dyed left hindpaw. The rat was trained to step on the tops of the hurdles. The circumference speed mentioned in this figure is measured at the top of the hurdles. For an explanation of the zones A, B, and C, see text.

after the injection of a ChE inhibitor, and the third test—at a 1.5 times higher circumference speed—immediately followed the second test.

A color TV camera was positioned axially and received a lateral image of the wheel and the rat. This image was electronically subdivided in three zones: A, B, and C (see Fig. 1B). The X and Y coordinates of the movements of the red left hindpaw were analyzed. A well trained normal (preinjection) rat shows a rather stereotyped behavior when walking in the wheel. It makes a small step with the red paw when that paw moves from one rung to another or a large step when the paw skips a rung. Small and large steps could be separated electronically. In both cases the red paw remains in zone B of Fig. 1B. A rat with a failing motor coordination, however, may slip with the red paw between the rungs, thus entering zone C. This was registered as a "wrong" step. In addition, drugged animals exhibit other types of behavior, like clinging to a rung or tumbling off. In such cases the red paw may enter zone A. This was registered as "other activities." Following the 90-sec test periods the cumulative number of small, large, and wrong steps, as well as the cumulative duration of each of these classes of activities was calculated. In addition, the cumulative time that the left hindpaw stayed on a rung was recorded. A detailed description of the electronics and computer analysis will be published elsewhere.

Automated open field. Movements of the albino rat in a black open field (1 × 1 m, with 25 cm high enclosing walls) have been measured by a combination of registration of capacitance changes to record vertical movements (Wolthuis *et al.*, 1975) and a TV video technique for recording horizontal movements (Tanger *et al.*, 1978). This method, which results in the registration of 11 classes of movements (see Fig. 5), has previously been used in our laboratory for studies on ageing (de Koning-Verest *et al.*, 1980) and on drug effects on learning (Wolthuis, 1981).

Active avoidance in an automated two-way shuttlebox. This technique has been described in detail elsewhere (Wolthuis, 1981). In essence, each animal received 20 trials a day at intervals of 1 min (±20% random). In each trial the rat had to learn to avoid footshock (250 μA, constant current principle) by moving into the other compartment within 10 sec after a light stimulus has been presented. Crossing from one compartment to the other was registered automatically by the interruption of infrared beams on both sides of the connecting door. The construction prevented that tail flicks interfered with the measurements. Criterion was reached when the animals had made 80% or more correct avoidance reactions (CAR's) in two successive sessions. This usually took 5 to 7 days. In the present experiments, the day after the criterion was reached the animals were tested 30 min after injection of a cholinesterase inhibitor.

Two compartment light–dark passive avoidance. By replacing one of the compartments of the above-mentioned shuttlebox by a black Plexiglas compartment of the same dimensions, inserting a guillotine door between the com-

partments and using a different program the shuttleboxes used were transformed into passive avoidance apparatus (see Wolthuis, 1981). One-trial training occurred by hand. The animal was placed in the illuminated compartment with its head facing away from the guillotine door. When the rat had entered the dark compartment the guillotine door was closed for 15 sec, during which period the animal received a scrambled footshock of 250 μA. Thereafter, the animal escaped from the dark compartment and was taken out after it had calmed down (approx 30 sec). Later, 24 hr, the animals were again placed in the illuminated compartment and during 30 min the following four parameters were automatically registered: (1) the latency until entering the dark box, (2) the cumulative time spent in the dark box, (3) the number of crossings, i.e., compartment changes, and (4) the number of approaches into the dark compartment. An approach—followed by a retreat—caused a different sequence of infrared beam interruptions compared with a crossing; this was electronically sorted out and registered.

Compounds were administered either 30 min before testing started or—in some cases—30 min before training.

Compounds and administration. TEPP (tetraethyl pyrophosphate), soman (*o*-pinacolyl methylphosphonofluoridate), and sarin (*o*-isopropylmethylphosphonofluoridate) were synthesized at the Prins Maurits Laboratory TNO by C. de Borst. Pyridostigmine bromide was obtained from Hoffman–LaRoche BV, at Mijdrecht, The Netherlands, and physostigmine sulfate from Nutritional Biochemicals Corporation, Cleveland, Ohio. The organophosphates were injected subcutaneously (sc) and the carbamates intraperitoneally (ip). In almost all cases, except in a few passive avoidance experiments, the ChE inhibitors were injected 30 min before testing started. Doses were never higher than 30% of the LD50 values.

Statistics. The open field data were analyzed according to Kruskall and Wallis (see Hollander and Wolfe, 1973), followed by the simultaneous statistical interference method of Dunn, adapted by Newman–Keuls (see Miller, 1966). The same methods were used for the acquisition experiments in the shuttlebox. For the passive avoidance test the data on "latencies" were analyzed according to Welch (see Natrella, 1963) and the data on "dark box time per 3 min" were analyzed with the method of Scheffé (see Miller, 1966). The data from the other tests were analyzed according to Welch. In all cases where the term "significant" is used, this means $p < 0.05$, two tailed.

RESULTS

Toxicity

The LD50 values of the ChE inhibitors tested are given in Table 1.

TABLE 1

	TEPP (sc)	Sarin (sc)	Soman (sc)	Pyridostigmine (ip)	Physostigmine (ip)
LD50 (μg/kg)	279	124	152	2699	1621
95% conf. lim.	253–324	116–131	143–166	2336–2876	1386–1947
Racetrack					
Long time interval	>30	>30	>30	>30	>30
Short time interval	>30	>30	>30	>30	>30
Walking test	>30	n.t.	>30	n.t.	n.t.
Hurdle-stepping test	>30	>30	30	10	3
Open field	>30	>30	3	5.5	4.5
Active avoidance	>30	>30	1[a]	10[b]	3.5[c]
Passive avoidance	n.t.	n.t.	n.t.	=30	10

	MED (%LD50)	Conf. lim.	μg/kg	
[a] Soman	0.91	(0.05–1.86)	1.35	sc
[b] Pyridostigmine	8.9	(6.8–14.6)	243	ip
[c] Physostigmine	3.5	(0.2–8.2)	56	ip

Note. The LD50 values of five cholinesterase inhibitors used and the dose levels—expressed as %LD50—at which these inhibitors had significant effects in the behavioral tests mentioned in the vertical column on the left side. The effective doses shown were estimates, except in the case of shuttlebox testing, where the minimal effective dose was calculated for three inhibitors. n.t. = not tested.

Racetrack

When animals had to make single runs just before, and 30, 60, and 90 min after administration of an ChE inhibitor, none of the compounds had any effects on run times at the highest dose level tested, i.e., 30% of the LD50. At this dose level equally negative findings were obtained with the "fatigue test," where each animal had to make five consecutive runs in a rapid sequence, 30 min after the injection of the ChE inhibitor. The negative result of one of these tests with physostigmine is shown in Fig. 2.

Walking Test

At 30% of the LD50, neither TEPP (see Fig. 3) nor soman affected the 12 parameters measured in the walking test. Therefore, the sensitivity of the test was investigated with acrylamide, apomorphine, Valium, alcohol, and

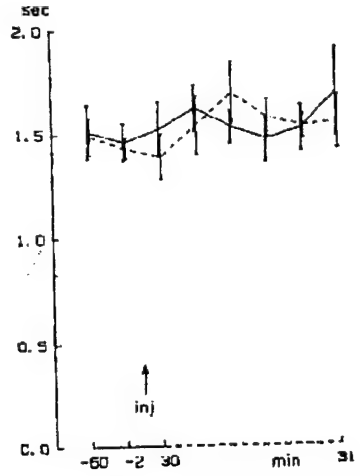


FIG. 2. Racetrack performance; the absence of an effect of 30% LD50 physostigmine ip in the mean (±SE) running time. Rats were trained during 3 days (three runs per day). On the fourth day they were tested 1 hr and 1 to 2 min before injection. Thirty minutes after injection they were tested in five successive runs immediately following one another. (——) saline (n = 8); (---) physostigmine (n = 8).

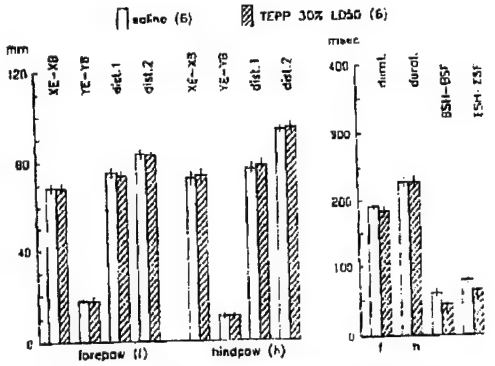
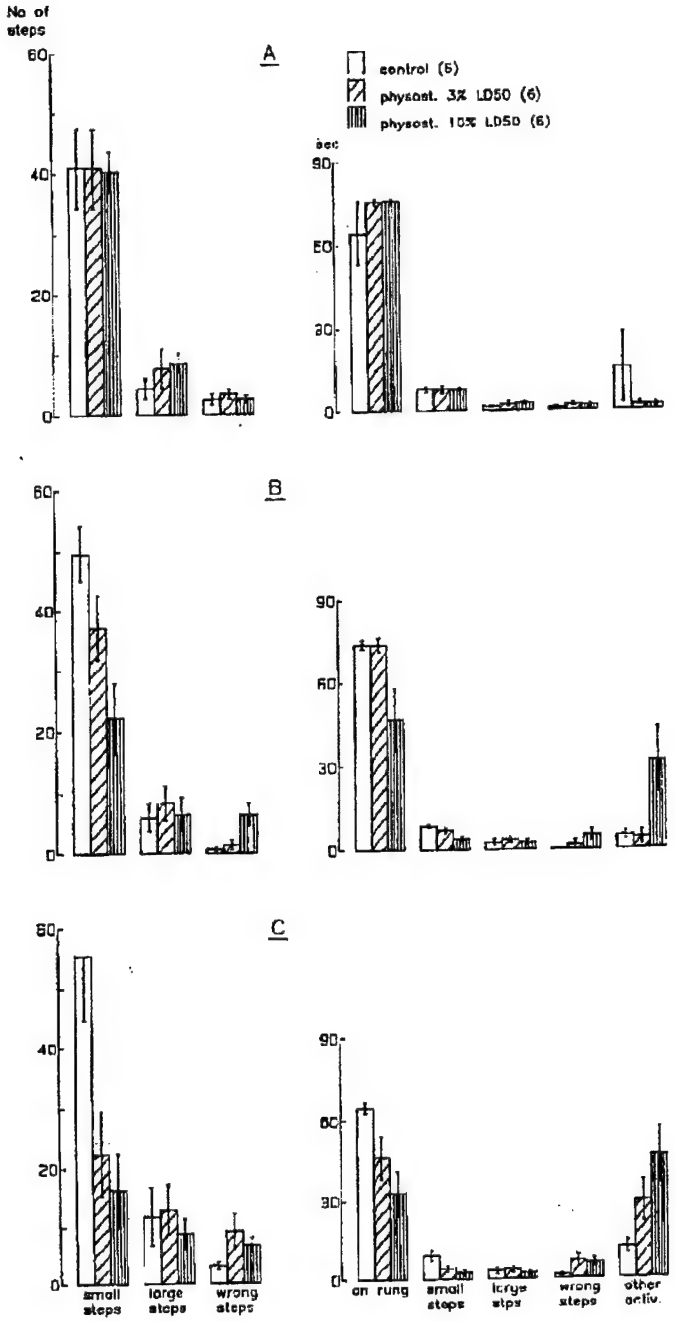


FIG. 3. Step analysis in the walking test: the absence of an effect of 30% LD50 TEPP. The X and Y coordinates of the moving left fore- and hindpaws were measured during 40 steps. XE–XB is the difference between the X-position at the beginning (XB) and end (XE) of each step; the same coding is used for the Y-coordinate. Dist. 1 is the shortest distance between take-off and touchdown point of each step and dist. 2 the actual length of the track that the paw followed. Durat. is the duration of the steps of the fore- (f) and hindpaw (h). Coordination between fore- and hindpaw is expressed as BSH–BSF, i.e., the time difference between the beginning of a step with the hindpaw (BSH) or the forepaw (BSF). The same is calculated for the end of a step with the forepaw (ESF) and the hindpaw (ESH). The number of animals per treatment group are shown in parentheses.

tremorine. The results showed a very high degree of reproducibility; effects of these compounds were only demonstrable at dose levels that also caused overt symptoms. Since the present study was preliminary aimed at doses of ChE inhibitors that did not cause overt symptoms, no further testing of the other inhibitors with this method was done.

Hurdle-Stepping Tests

This type of acquired behavior appeared to be sensitive to carbamates. At the low speed of 5.4 cm/sec physostigmine (see Fig. 4B) caused a dose-dependent reduction of the number of correct small steps. The number of wrong steps, the time that the hindpaw remained on a rung and the "other activities" were only affected at a dose level of 10% LD50. At the higher speed of 8.2 cm/sec (see Fig.


No of steps
A
control (6)
physost. 3% LD50 (6)
physost. 10% LD50 (6)
sec
B
C
small steps
large steps
wrong steps
on rung
small steps
large stps
wrong steps
other activ.

4C) the effects were more pronounced and then physostigmine also caused significant effects at 3% LD50. Pyridostigmine, in doses up to 30% LD50, did not have any effect on this type of complex stepping behavior at low circumference speed. However, with increased circumference speed, pyridostigmine caused a dose-dependent reduction of the number of correct small steps, an increase in the number of wrong steps and "other activities." The lowest dose of pyridostigmine that caused a significant effect was approximately 10% LD50. TEPP and sarin were not effective in doses up to 30% LD50. Surprisingly, soman also caused no changes in doses up to 30% LD50. This ill-understood negative finding led to an extensive search to find an explanation (see last section of Results).

Open Field Tests

A number of parameters in the open field test were affected by low doses of soman, pyridostigmine, and physostigmine, whereas TEPP and sarin did not influence open field behavior in doses up to 30% of their LD50 value (see Table 1). Interestingly, the lowest dose of the carbamates particularly affected the parameters "distance run"—as a measure of activity—and "entries corners"—as a measure of exploratory behavior—whereas the lowest dose of soman typically had an effect on the small movements of the animal (amplitude 2, see Fig. 5) and influenced the larger movements (amplitude 3) as well as rearing in a dose-dependent manner. Dose-dependent effects on distance run, entries corner, or entries inner field were also observed, but on these parameters the lowest dose of 3% LD50 of soman had no effect. There was also a small, dose-dependent reduction of respiration rate; at a dose of 30% LD50 soman these effects became significant (see Fig. 5). This effect does not necessarily indicate that respiration had slowed down; it could also mean that respiration had become more superficial so that part of the respiratory movements fell below the detection level.

Experiments with the Two-Way Shuttlebox

This active avoidance technique appeared to be sensitive to the effects of soman, physostigmine, or pyridostigmine, whereas it was not sensitive to the effects of TEPP or sarin (see Table 1). As an example, Fig. 6 shows that sarin in doses up to 30% LD50 had no effect on shuttlebox performance, whereas soman in doses as low as 1% causes a significant decrement in performance. In Fig. 7 the dose-related performance decrement caused by soman and the absence of effects of sarin and TEPP are shown, expressed as percentages of the performance of control animals. Similar examples for the effects of physostigmine and pyridostigmine are shown in the Figs. 8 and 9.

Passive Avoidance Experiments

The effects of physostigmine and pyridostigmine on the different parameters of a passive avoidance task are shown in Fig. 10. The animals were tested for retention on the day following one-trial learning, 30 min after an injection with carbamates. The lowest dose of

FIG. 4. The hurdle-stepping test: effects of 3 or 10% LD50 physostigmine i.p. (A) Shows the mean (±SE) results obtained before injection. (B) The results 30 min after injection. (C) The results obtained immediately after B, but with the circumference speed of the wheel increased by a factor 1.5×. All tests lasted 90 sec. At low speed (B) a dose-dependent reduction of correct small steps is seen. In addition 10% LD50 caused a significant increase in the number of wrong steps and the time spent on "other activities" as well as a significant decrease of the time that the hindpaw stays on a rung. At a higher circumference speed the effects were more pronounced: physostigmine then caused significant effects at 3% LD50. The number of animals per treatment group are shown in parentheses.

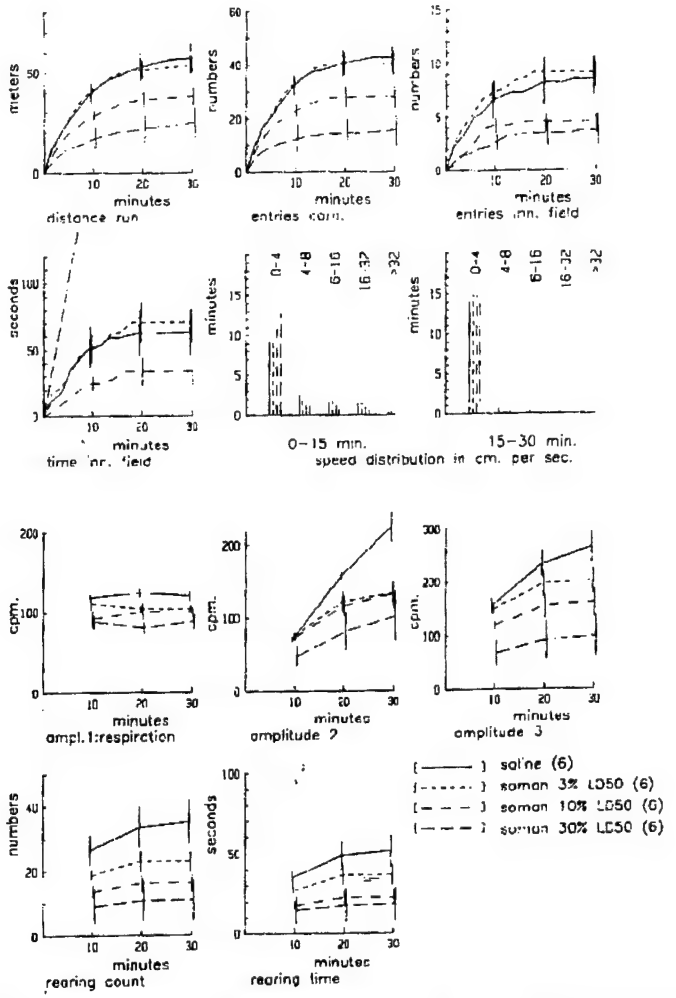


FIG. 5. The open field test: effects of different doses of soman measured 30 min after the injection. All graphs, except those on respiration, represent mean (±SE) cumulative data. Top row: distance run in meters (left), the number of entries into corner sections of 20 × 20 cm (middle) and the number of entries into the inner field of 60 × 60 cm (right). Second row: time spent in the inner field (left) and the time that movements, subdivided in speed classes, were sustained during the first and second 15-min periods of testing (middle and right, respectively). Third row: respiration in counts per minute (cpm) (left) and the number of small and large movements (middle and right, respectively). Bottom row: the number of rearings and the time spent in an upright position, respectively. The number of animals per treatment group are shown in parentheses.

physostigmine that induced a significant change in at least one of the parameters was 10% LD50, the lowest effective dose of pyridostigmine was 30% LD50. Since these results indicated that—tested in this way—the passive avoidance task was not very sensitive to the effects of ChE inhibitors, experiments were performed in which the carbamates were administered 30 min before the learning trial. This provided no additional information: only

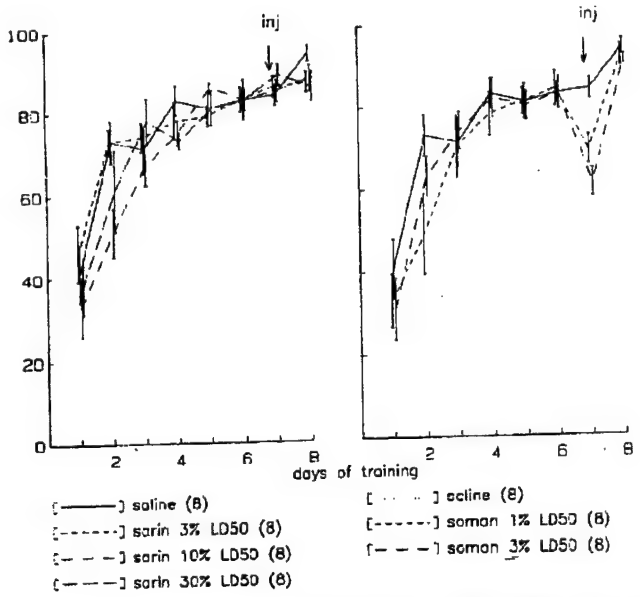


FIG. 6. Active avoidance in a two-way shuttlebox: the effects of different doses of sarin (left) or soman (right) on performance. Note that sarin in a dose of 30% LD50 had no effect whereas soman caused significant decrement of performance in a dose of 1% LD50. The data are presented as mean ± SE, the number of animals per treatment group are shown in parentheses.

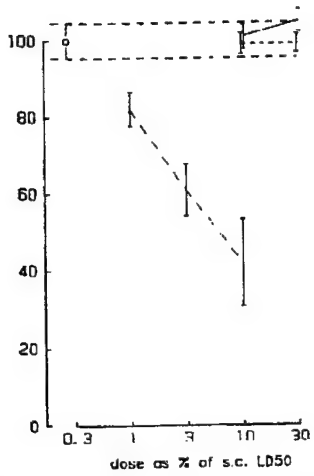


FIG. 7. The dose-related decrement of performance by soman. Sarin and TEPP are ineffective in doses up to 30% LD50. The mean (±SE) values are expressed as percentages of the performance of control animals. (O) saline (n = 30); (- - -) TEPP (n = 18); (——) sarin (n = 16); and (— — —) soman (n = 36).

30% LD50 of physostigmine caused—a rather inconsistent—effect.

Tolerance to the Behavioral Effects of Soman

As mentioned under the section "hurdle-stepping tests," it came as a surprise that soman did not affect motor coordination in this acquired task. A systematic search for an explanation of this unexpected negative result included checks of purity of the soman (which appeared to be better than 99%), factors such as the time of day, changes in breeding regimen of the rats, etc. A rather sudden change must have taken place, since from that moment on shuttlebox performance also was unaffected by soman, even in doses of 30% LD50 (Fig. 11). In contrast, physostigmine caused the same or an even more pronounced decrement of shuttlebox performance (Fig. 11). These results were confirmed several times. Since the

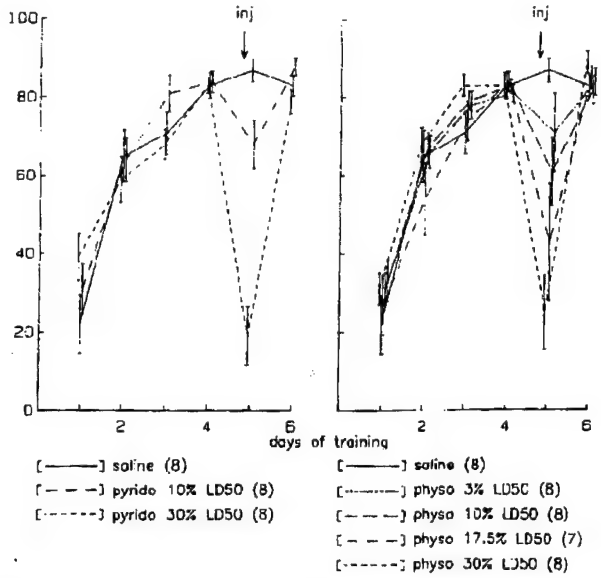


FIG. 8. The effects of pyridostigmine (left) and physostigmine (right) on the performance of an active avoidance task in a two-way shuttlebox. Compare with Fig. 6.

firm supplying the food pellets had switched from one grain producer to another one, it was thought possible that by induction of liver microsomal enzymes the metabolism might have changed. This assumption was strengthened by the finding that pentobarbital-induced sleeping time was slightly, but significantly shorter in animals raised with the "new chow" compared with animals raised with "old chow." In terms of ChE inhibitors, it might have been that the "new chow" had caused a substantial increase in the amount of aliesterases in rat blood. Since soman does and carbamates do not bind to aliesterase, a higher level of blood aliesterase might effectively have removed the small amounts of soman before it reached the sites of action in the CNS, whereas the effects on the carbamates remained unchanged. This notion fitted in with the above mentioned findings in the hurdle-stepping test and those of the recent shuttlebox experiments, but had to be rejected when it appeared that aliesterase levels in the blood of these rats did not differ from those determined several times during the past years. These determinations were carried out by Dr. L. P. A. de Jong from the Prins Maurits Laboratory TNO.

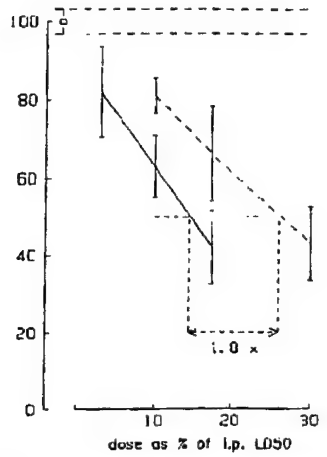


FIG. 9. The dose-related decrement of shuttlebox performance caused by two carbamates. The means (±SE) are expressed as a percentage of the performance of control animals. (O) saline (n = 16); (——) = physostigmine (n = 39); and (– – –) pyridostigmine (n = 40). Compare with Fig. 7.

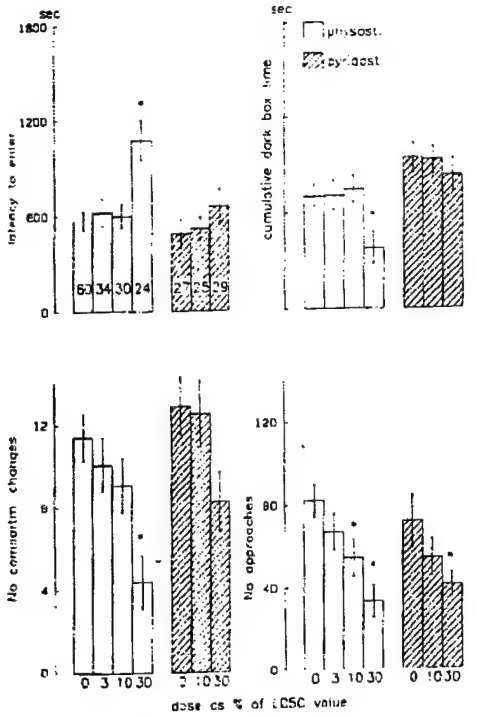


FIG. 10. The effects of two carbamates on retention in a one-trial light/dark discrimination passive avoidance task. The carbamates were injected 30 min before testing. The most sensitive parameter appeared to be the number of approaches per 30 min. The number of animals per treatment group are shown in the diagrams of the upper left graph. The graphs show the mean (±SE) values.

That indeed a factor in the food was responsible for the absence of the effects of soman could be shown in an experiment in which groups of animals were raised from conception onward with "new chow" and "old chow," respectively. Performance in the shuttlebox was only affected by soman in animals raised with "old chow" (Fig. 12).

Since the negative results with soman in the "new-chow" animals might be explained by development of tolerance due to presence of higher amounts of organophosphate pesticides in the "new chow," attempts were made in the Prins Maurits Laboratory TNO to determine these agents in "old-" and "new chow." In samples from both chows approximately equal amounts of organophosphates were

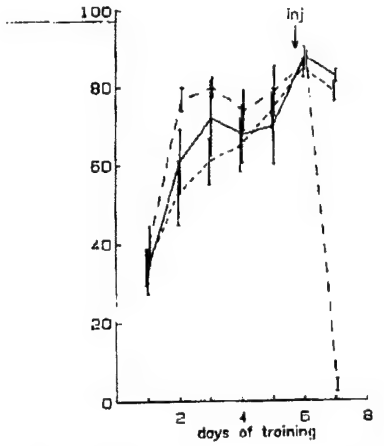


FIG. 11. The effect of physostigmine (10% LD50, sc) and the absence of the effect of soman (30% LD50, sc) on shuttlebox performance of rats raised with "new-chow." (——) saline (n = 10); (---) soman (n = 10); and (– – –) physostigmine (n = 10). The vertical bars represent SE.

found. Unfortunately, the compounds could not be identified. If substantial tolerance to soman would have developed, an increased LD50 for soman should be expected. This, however, was not found. Thus, the puzzle remains to be solved.

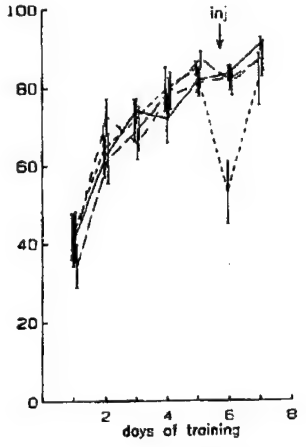


FIG. 12. The effect of saline or soman (10% LD50) on shuttlebox performance of rats raised from conception onward with "old-chow" or "new-chow." The mean (±SE) values are shown. (——) old-chow saline (n = 13); (---) old-chow soman (n = 12); (– – –) new-chow saline (n = 12); and (— —) new-chow soman (n = 13).

Inhibition of Blood ChE by Carbamates

During the same period in which the behavioral experiments were carried out, measurements of blood ChE activity were performed in carbamate-injected rats of the same strain, age, and sex. These experiments were done for different reasons under supervision of Dr. F. Berends, who granted permission to present the data.

It appeared that upon ip injection of physostigmine (10 μg/kg) a peak of 30 to 40% inhibition of total blood ChE was obtained in 20 min, followed by a spontaneous reactivation in the subsequent 120 min. With pyridostigmine, this inhibition level was obtained with a dose of 12 μg/kg ip and graphically there was a plateau rather than a distinct peak. With this inhibitor spontaneous reactivation of total blood ChE took more than 5 hr.

DISCUSSION

One of the main questions in these experiments was whether, as a result of exposure to low doses of ChE inhibitors, behavioral disturbances would be detectable in rats without overt symptoms. The answer to this question was positive: doses of soman, physostigmine, or pyridistigmine that caused no overt symptoms and did not affect physical performance in a racetrack or during reflex-like walking, disturbed an acquired hurdle-stepping task, open field behavior, and active as well as passive avoidance behavior (see Table 1). TEPP and sarin did not induce behavioral changes in doses up to 30% LD50 that did not cause overt symptoms. Most likely this could be explained by the fact that both these ChE inhibitors, in contrast with soman, have a predominantly peripheral mode of action (Wolthuis, 1981). Besides sarin, the organophosphate TEPP was included in the investigation, because after sarin intoxication a rather rapid spontaneous reactivation of AChE takes place (Meeter and Wolthuis, 1968), which might make interpretation of the results difficult. It came as a surprise that pyridostigmine, allegedly an inhibitor that hardly passes the blood-brain barrier (Goodman and Gilman, 1970), caused behavioral disturbances in relative low doses. In three of the four tests (see Fig. 1 and Table 1) where carbamates were effective, the lowest dose of pyridostigmine that caused a significant effect was only 2 to 3 times higher than that of physostigmine (expressed as percentages of the LD50 values). This was also the case of the results were expressed as a percentage of the dose needed to cause an inhibition of 30 to 40% of the total blood ChE. This small difference indicates that a larger portion of pyridostigmine passes the blood-brain barrier than hitherto accepted. Since carbamates are proposed as prophylactic agents against intoxication with organophosphate ChE inhibitors, this finding may explain why in animals pretreatment with pyridostygmine protects against intoxication with soman (see, e.g., Inns and Leadbeater, 1983), an inhibitor with its predominant action in the CNS (Wolthuis *et al.*, 1981). So far, it had been difficult to understand how a carbamate that did not enter the CNS could protect against an OP that acted predominantly in the CNS.

In the earlier experiments soman reliably caused a significant decrement of shuttlebox performance at dose levels lower than 1% LD50 (=1.5 μg/kg). This was repeatedly found and there can be no doubt that this was a real effect that could be ascribed to a central effect of soman. The finding that in the last experiments the behavioral effects of soman could not be elicited even after doses of 30% LD50 still goes unexplained. The results of subsequent experiments indicated that a factor present in the new batch of rat chow was responsible. Various speculations with respect to this factor are still possible, but it should be kept in mind that if this was a form of tolerance it was a rather strange one: the effects of carbamate were not changed and the LD50 value of soman was not increased. That tolerance to ChE inhibitors exists, is amply proven (for references, see Introduction). In the present experiments the behavioral effects of soman had also disappeared when the an-

imals were retested 24 hr after injection. It was very unlikely that in such a short period spontaneous reactivation had occurred or sufficient new cholinesterase had been synthesized to replace the amount of enzyme inhibited by 30% LD50 soman.

The types of tests that were most sensitive to the effects of these ChE inhibitors were the open field test and active avoidance in the shuttlebox. A passive avoidance test was not very sensitive and, in contrast to effects obtained with parathion (Reiter *et al.*, 1973), administration of physostigmine before training in a few additional experiments showed no effect at lower dose levels. Step analysis of reflex-like moving in the walking test appeared insensitive to doses of various neurotoxic agents and also to ChE inhibitors that did not cause overt symptoms. In contrast, the acquired task of moving in the hurdle-stepping test appeared quite sensitive to carbamates and in very recent preliminary experiments also to low doses of diazepam. It may be that the explanation for the difference between the sensitivity of the walking test and the hurdle-stepping test, where demands are made on the same elements of the motor system, has to be found in the conditioning necessary to reach a desired performance level in the hurdle test. It is plausible that the activity of higher CNS centers during acquisition of this task is primarily responsible for the increased sensitivity of the hurdle-stepping test.

In conclusion, the results of these experiments indicate that (1) after exposure to low doses of ChE inhibitors acute behavioral effects may occur without overt symptoms, and without disturbance of the physical fitness of the subject, (2) the individual characteristics, particularly the relative efficacy of the inhibitors in passing the blood-brain barrier play a decisive role in the occurrence of the behavioral effects, and (3) large differences exist between the sensitivity of the six test procedures applied. The results suggest that tests that require motor activity and at the same time make a demand on the function of higher CNS structures are most sensitive.

ACKNOWLEDGMENTS

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Pyridostigmine Bromide Alters Locomotion and Thigmotaxis of Rats: Gender Effects

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HOY, J. B., B. A. CODY, J. L. KARLIX, C. J. SCHMIDT, I. R. TEBBETT, S. TOFFOLLO, F. VAN HAAREN AND D. WIELBO. *Pyridostigmine bromide alters locomotion and thigmotaxis of rats: Gender effects.* PHARMACOL BIOCHEM BEHAV 63(3) 401–406, 1999.—Male rats and female rats in the proestrous and metestrous stages of estrus were tested to determine the effects of pyridostigmine bromide on locomotion rate and thigmotactic response using doses of 3.0, 10.0, and 30.0 mg/kg. Thirty minutes after administration of the pyridostigmine bromide the rats were videorecorded for 2 h in a 1 m^2 open-field arena. The rats' activities were analyzed for the drug's effect on speed throughout the 2 h and during six 20-min segments. Also, the times that the rats were observed moving through the central 50% of the arena were determined. Locomotion rates decreased significantly, and thigmotaxses increased significantly in all groups of rats as a dose response to pyridostigmine bromide. Habituation occurred over 2 h for both responses, primarily during the first 40 min. Female rats were more affected than males, but metestrous and proestrous females did not differ significantly in their responses. At the 30 mg/kg the effect was persistent throughout the test period. Proestrous females dosed at 30 mg/kg had much higher pyridostigmine bromide serum levels than metestrous females and males. © 1999 Elsevier Science Inc.

Pyridostigmine bromide Locomotion Open field Thigmotaxis Gender effect

PYRIDOSTIGMINE bromide (PB) is an acetylcholinesterase inhibitor that has been used as a treatment for myasthenia gravis for many years (8). A recent study has shown a synergistic effect between DEET and both PB and permethrin when administered to cockroaches (15). Coexposure to PB, *N,N*-diethyl-*m*-toluamide (DEET), and permethrin has also been shown to have synergistic behavioral effects in chickens (1). A synergistic effect (LD_{50}) of coexposure to PB, DEET, and permethrin using male rats has been reported (12). In this case, oral administration of PB in propylene glycol resulted in estimation of LD_{50} of 61.6 mg/kg. Combinations of the three drugs at dosages calculated to cause mortality of 48% of the animals caused mortalities of 80 to 90%.

Sublethal effects of neurotoxic compounds may be seen in various measures of locomotor activity (4,9,14). Neurobehavioral screening of pesticide effects on mammals has been reported (13). Low doses of PB (3–12 mg/kg) decreased response frequency during operant tests (17). Gender and estrous cycle were identified as factors in reduced open-field activity produced by interleukin-1b (2). Similarly, gender differences in susceptibility of cockroaches to toxicants has been reported (10). Open-field locomotor activity in rats, using automated data acquisition, can show chemically induced changes in speed and thigmotactic responses (3,4,9,16). Significant changes in the open-field behavior of rats dosed with PB at 5.5% of LD_{50} have been reported (19). In this case, intraperitoneal administration of PB resulted in estimation of LD_{50} at 2699 mg/kg.

The purpose of this study was to determine the effects of PB on locomotor and thigmotactic activity of male rats and female rats in proestrous and metestrous. Furthermore, we sought baseline information for future study of the synergistic effects of PB, DEET, and permethrin on locomotion.

METHOD

Subjects

Sprague–Dawley rats (250 g) were obtained from Harlan–Sprague–Dawley (Indianapolis, IN), and housed same sex, two per cage, under a reversed light cycle of 12 D:12 L (lights

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on 1800 h), and fed rat chow ad lib. The rats were identified by ear-punch code. Each rat was handled about 30 s 5 days/week for at least 7 weeks prior to testing. Treatments were assigned to individuals at random within groups and time of test. Tests were done between 900 and 1700 h. Male, and proestrous, and metestrous female rats were tested two at a time in individual arenas. Male rats were treated first, and whenever possible metestrous females were tested second and proestrous females last. Alternatively, only females of one type were tested if both types were not available on a given day. All dosing and handling of test subjects were done by the same technician.

Estrous Stage Determination

Female subjects were examined 1 to 3 h before testing to determine their estrous cycle status. The criteria for assignment to proestrous or metestrous categories was based on microscopic examination of epithelial cells found in the vaginal fluid of the rats.

Drug and Dosage

PB obtained from Sigma (St. Louis, MO) was orally administered by gavage tube in distilled water at low, medium, and high doses, 3.0, 10.0, and 30.0 mg/kg, respectively, in a volume of 5 ml/kg. Control animals were dosed with matching volumes of distilled water. Test subjects were held 30 min prior to introduction to the test arenas, then placed in the center of the arena about 30 s prior to recording of their activity.

Arenas

The tests for locomotor activity were done in two black ABS plastic arenas that were 100 × 100 × 30-cm high. Each arena was surrounded by a black curtain. The arenas were on opposite sides of a rack that supported lights, video cameras, and video cassette recorders. Indirect low intensity light was provided by three 60-watt red bulbs approximately 2.2 m above each arena, and located so that the center of each arena received about 2 lx and the corners received 1 to 2 lx. Prior to use, feces and urine were removed and each arena was swabbed down with about 10 cc of 80% ethanol solution, and wiped dry with paper toweling. The air-conditioned testing room was maintained at approximately 22°C. The arenas were in a locked room well insulated from outside sounds. Within 1 min of the start of each test the experimenter left the room for the remainder of the automatically recorded 2-h test.

Recording

Horizontal locomotion was recorded using a Topica (model TP-505D/3) CCD video camera and a Sharp (model XA-601) video cassette recorder. Paralax was minimized by mounting the cameras 2 m above the arenas. The 1 m^2 arena was visualized as 240 × 240 pixels. Therefore, a movement over 24 pixels was a move of 10 cm. A speed of 30 pixels/s was about one rat body length/s, or 7.5 m/min. Raw data recorded in pixels/s were converted to m/min before data analysis was completed. All video records were archived following computer analysis.

Locomotor Analysis

Locomotor activity was quantified using Apple Power Macintosh-based software and a Macintosh (model 7100/80 with an AV board installed) (6,7). The software calculates the center of mass of the rat. To avoid including the rat's tail in determining the location, or movement; India ink was applied to the tail prior to PB administration. Each 2-h recording was reduced to an ASCII file of observations at 1-s intervals that represented both the positions of the subject on a 240 × 240 pixel grid (X,Y coordinates) and the running average of locomotion rate over five observations. Sampling at 1-s intervals filtered out recording of short-range stereotypic movement that would otherwise have been scored as locomotion. The raw data were used to calculate speeds for each second of the record, which were then used in lieu of the running average provided by the original analysis.

The number of times that the subject was recorded in the center 50% of the arena was filtered so that only those times that the subject was moving faster than 1.2 m/min (2 cm/s) were counted. That filter excluded observations that might have occurred if a subject had become inactive, thereby avoiding a high center zone score for a subject that had collapsed in midarena.

The ASCII file for each subject was then imported into StatView and further analyzed for locomotion rate and thigmotatic response in six 20-min bins of the 2-h test period.

Blood Serum Analysis

An estimate of the serum level of PB in an individual rat at the beginning of the test period was obtained by waiting at least 5 days after a given rat's locomotion test and taking a blood sample by decapitation 30 min following a second similar dose and anesthesia with methoxyflurane. Female subjects were dosed the second time during the appropriate stage of the estrous cycle. Three milliliter blood samples were kept on ice for 2 h, centrifuged, serum drawn off, and frozen at −70°C. The serum was then analyzed for PB as follows: the serum sample was transferred to a stoppered tube and vortexed with 1 ml of 0.025 M potassium phosphate buffer at pH 3. This mixture was then applied to a Strong Cation Exchange column that have previously been conditioned under vacuum on a Vac Elut manifold (Varian) with methanol, water, and 0.025 phosphate buffer. After application of the sample, the column was air dried for approximately 30 s and then washed with phosphate buffer and 0.1 M acetic acid. The column was again air dried for 30 s before eluting off the adsorbed drugs with 3% ammoniacal methanol. The final extract was evaporated to dryness under nitrogen and the residue reconstituted in 50 μl of methanol. A 20-μl aliquot of the extract was then used for HPLC analysis. This analysis was performed using a Waters 510 pump to deliver solvent at 1 ml/min to a Hypersil 5 μm ODS column. A Waters C18 Guard Pak precolumn was used to protect the analytical column. The detector was a Waters 486 variable wavelength detector set at 272 nm with a Dell 486 data system and Millenium (TM) software. The mobile phase consisted of acetonitrile–0.1% triethylamine in water (adjusted to pH 3.2 with phosphoric acid. 70:30). Quantitative analyses were achieved by comparison of peak areas with unextracted standards. Each determination was taken as the mean of three replicate injections. The calibration graph was produced over the range of 0.05–5 μg/ml.

Experimental Design

The experimental design was three groups of rats × four application rates × 10 subjects for each application rate. Space limitations in the rat colony required that the rats be tested in two batches, 20 males and 40 females each, for a grand total of 120 rats. Each batch was tested over a 15–22-

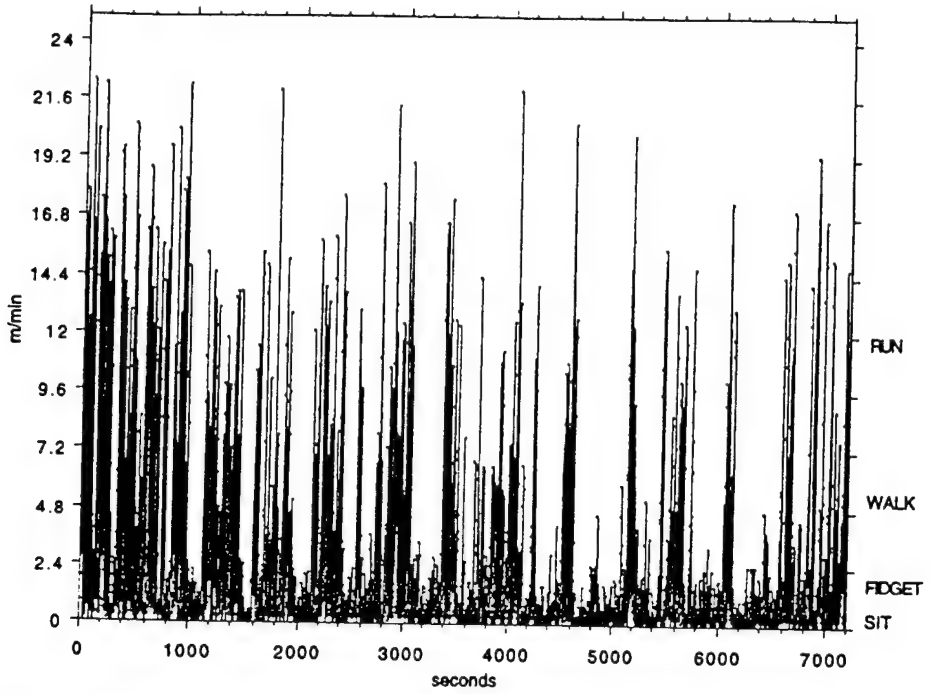

FIG. 1. Typical range and variation in locomotion (m/min) during a 2-h period following administration of vehicle. Note the indications of the type of behavior associated with various speeds at the right margin of the figure.

day period, with 29 days between batches. The data from the two batches were pooled.

Statistical Analysis

Differences between locomotion rates and counts of observations in the center zone of the arena were determined by repeated-measures ANOVA (group × time), for the total 2 h observation time. Comparisons between groups were then done using Duncan's Multiple Range Test ($p < 0.05$). Subsequently, post hoc power calculations were done with assumptions of higher alpha levels using GPOWER (5).

RESULTS

Locomotion rates as high as 30 m/min were observed. Figure 1 illustrates the range and variation of locomotion rate for a typical rat dosed with vehicle. Sitting, fidgeting, walking, and running fall into the progressively higher ranges indicated in the figure. The ranges of speed associated with these activities were subjectively determined, and are provided as a general indication of the alternation of activities over the observation period. Also, Fig. 1 shows a trend toward fewer and shorter peaks throughout the 2-h period as well as the rapid changes in speed.

Locomotion Rate

Habituation of the locomotion rate, as suggested by the reduced number of peaks over time, is more clearly illustrated by the mean speeds found in each successive 20-min period of observation. Figure 2 shows the habituation curves for all groups and treatments of rats. Each group and treatment followed the same pattern, i.e., a rapid decline in mean speed during the first hour, followed by very little change in mean speed during the second hour. The dose effect of PB can also be seen in this figure.

Figure 2 shows locomotor activity (speed in mean m/min) during 20-min segments of the experimental session for male rats and female rats in either proestrous or metestrous phase of the estrous cycle following the administration of vehicle or PB. ANOVA revealed a significant three-way interaction among time of observation, dose, and gender, $F(30, 535) = 1.72$, $p < 0.03$. This figure shows that for subjects given the vehicle speed decreased from an initial high of about 4 m/min to about 1.75 m/min during the final 20 min of the session. ANOVA revealed that the speed decreased as a function of dose, $F(3, 107) = 34.80$, $p < 0.01$. Post hoc analyses showed no significant differences between the administration of vehicle and 3 mg/kg PB, but that the speeds observed after administration of 10 mg/kg PB and after 30 mg/kg PB were significantly lower than vehicle. Planned contrast analyses at each time of observation (Table 1) showed that there were no significant differences between vehicle and 3 mg/kg PB in any of the groups of subjects. Significant differences were observed at all times of observation when the behavioral effects of vehicle were compared to those observed after administration of 10 mg/kg in metestrous and proestrous females. However, in male rats the decrease in speed after 10 mg/kg PB was only significant at time point 2. Planned contrast analyses showed that speed decreased significantly compared to vehicle administration in all groups of subjects after the administration of 30 mg/kg PB.

Center Zone Activity

The distribution of activity within the 1-m² arena favored the marginal area in all cases. That bias is illustrated in Fig. 3,

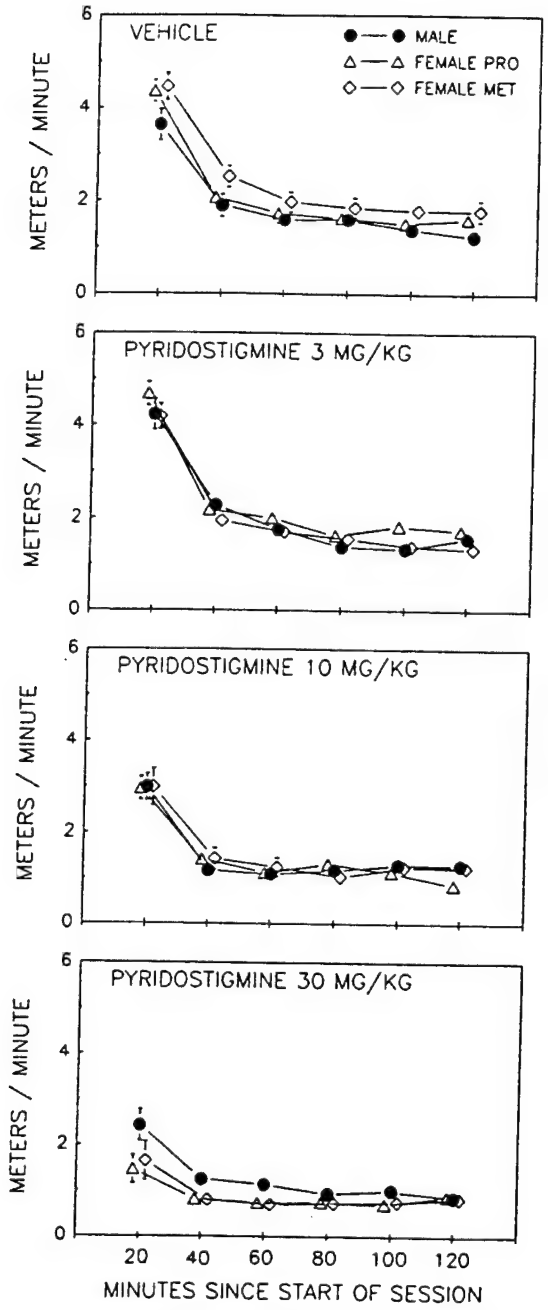

FIG. 2. Mean locomotion rates (m/min) in 20-min segments of 2-h observation periods according to rate of administration of pyridostigmine bromide.

which shows typical traces of the paths of rats given the four treatments used in our study. We quantified the distribution of activity by calculating the percent of the total observations in which the rat was observed moving through the central 50% of the arena. A dose effect was found in all groups of rats.

Figure 4 whose center zone activity for male rats, proestrous female rats, and metestrous female rats following administration of vehicle and 3, 10, or 30 mg/kg PB vs. vehicle.

TABLE 1

EFFECTS OF PYRIDOSTIGMINE BROMIDE (10, 30, AND 10 mg/kg vs. 30 mg/kg) ON LOCOMOTION RATE BY TIME PERIOD OF MALE, AND PROESTROUS AND METESTROUS FEMALE RATS

	Dose (mg/kg)								
	Males			Proestrous Females			Metestrous Females		
Pd.	10	30	10 vs. 30	10	30	10 vs. 30	10	30	10 vs.30
1	NS	33	NS	28	59	*	36	60	*
2	34	34	NS	28	60	*	50	68	NS
3	NS	NS	NS	30	56	*	37	65	*
4	NS	NS	NS	19	48	*	49	62	NS
5	NS	42	NS	26	41	*	28	57	*
6	NS	30	NS	40	35	NS	33	54	NS
Tot	NS	33	NS	30	50	NS	40	62	*

*Significant effects (alpha = 0.05) are indicated by the percent reduction from the control mean for comparisons in the first two columns. An asterisk indicates a significant difference where the effect of 10 mg/kg vs. 30 mg/kg is compared.

This figure shows that subjects tended to spend between 20 and 25% of the session time in the center of the arena following vehicle administration. PB dose dependently decreased the percentage of center zone observations, $F(3, 107) = 28.85$, $p < 0.01$. After the administration of 30 mg/kg PB subjects were in the center zone in less than 10% of the observations. Gender differences or interactions between dose and gender were not found.

Blood Serum Analyses

Postreatment analyses indicated that serum levels of PB for the three test groups were higher, but not proportionately

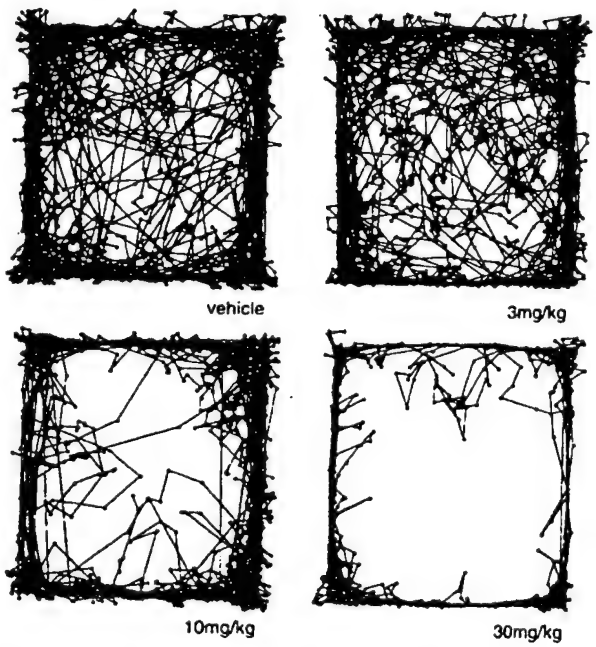

FIG. 3. Typical traces of the paths of male rats during a 2-h observation period, according to the indicated rate of administration of pyridostigmine bromide.

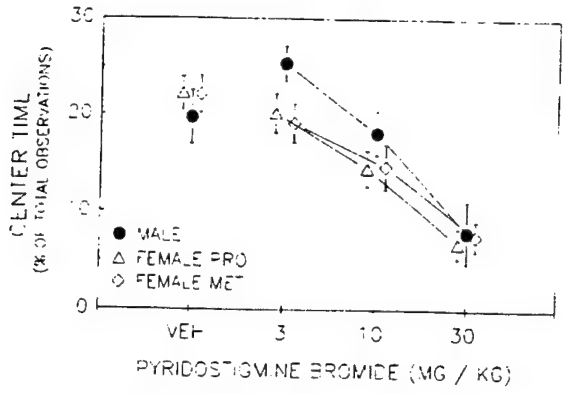

FIG. 4. Center zone time (the average percentage of the total number of observations that the subjects were in the center area (50%) of the arena ±1 SEM) for the groups of male and (proestrous and metestrous) female rats following administration of the vehicle and 3, 10, or 30 mg/kg of pyridostigmine bromide.

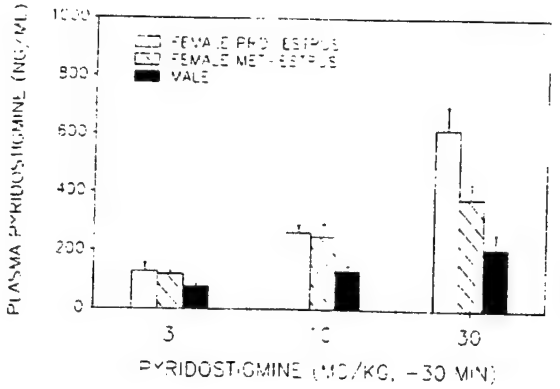

FIG. 5. Serum levels of pyridostigmine bromide (mean nanograms per ml, =1 SEM) observed after the second administration of 3 mg/kg left-most bars), 10 mg/kg (middle bars), or 30 mg/kg (right-most bars).

higher, with increased dose, i.e., a negatively accelerating dose–response curve. Figure 5 shows serum levels of PB observed 30 min after the second administration of 3, 10, or 30 mg/kg. A total of 70 observations figured in this analysis. No PB was found in control animals. The serum levels differed by dose. A significant interaction between PB dose and gender, $F(4, 61) = 5.64$, $p < 0.01$, and subsequent post hoc analyses supported the observation that PB levels in males differed only when compared after 3 and 30 mg/kg PB. In metestrous females, all three doses differed from one another, whereas in proestrous females differences were observed when 3 and 30 mg/kg and 10 and 30 mg/kg were compared, but not when 3 and 10 mg/kg were compared.

DISCUSSION

We have presented our locomotion data in terms of speed in m/min. The observed speeds correspond to the following types of activity, and provide an illustration of the types of behavior seen. A mean rate of less than 1.2 m/min indicated a sluggish rat moving less than 0.2 body length/s. Fidgeting or grooming behavior was recorded as movement less than 2.4 m/min (less than 0.5 body length/s). Walking resulted in a mean speed of less than 7.2 m/min (less than 1.5 body lengths/s). Running resulted in speeds ranging from 7.2 m/min to more than three times that rate.

We found gender differences and PB effects on locomotion rate. A previous study on male rats ($n = 6$) found no effect on the running speed in an open-field test following intraperitoneally administered PB at less than 10% of the LD_{50} (14). Recently, hens ($n = 5$) given 5 mg/kg PB orally for 60 days showed no locomotor effects (1). However, both studies lacked the power needed to find anything less than a catastrophic effect. In another study of PB effects on locomotion, male rats administered pyridostigmine and running on a treadmill became exhausted more rapidly than controls (11). Our findings that female rats were more sensitive than male rats, and the somewhat limited power of our test, suggest that additional tests using female rats in numbers adequate to balance type I and type II error are needed to find or rule out subtle effects. The problem of finding effects on sensitive but rare individuals within a population should also be addressed.

Center zone activity has been found to be a more sensitive measure of intoxication than speed when a stimulant was the toxicant (3,18). We found that PB depressed both measures, but we are not convinced that one measure is more sensitive than the other in our study. Separating the possible interaction of the two is outside the design of this study.

Serum levels of PB were higher in female rats than in male rats. In female rats they were also higher during the proestrous than during the metestrous phase of the cycle. These observations suggest that PB kinetics (liver metabolism and/or urinary excretion) may be modified by circulating gonadal hormones. At present, it is not known what mechanisms might be involved, but such warrants further investigation.

Table 1 shows the percent reduction from control level of locomotion rates for all cases significant at the 0.05 level. The contrast between the sexes is striking, with little effect observed in males. And, although ANOVA failed to show a significant difference between groups of females, the metestrous females quite consistently showed a greater reduction than the proestrous females.

Sublethal behavioral effects, by definition, are more sensitive and more relevant to drug safety than LD_{50}, or even an LD_1. The gender effect in rats is certain, with timing relative to estrous cycle a possible exacerbating factor in the toxicity of PB to females. If humans in general, and females at a crucial point in their menstrual cycle in particular, are more sensitive to PB than rats, the changes in rat locomotor behavior that we have found may be relevant to the clinical use of PB.

ACKNOWLEDGEMENTS

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The effects of pyridostigmine bromide on progressive ratio performance in male and female rats

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Abstract

Small doses of pyridostigmine bromide (PB) affect the acquisition and maintenance of food-maintained behavior in laboratory animals. The present experiment was designed to investigate the effects of this drug on food motivation. Male and female rats were trained to respond on a progressive-ratio schedule of reinforcement and treated with different doses of PB. PB dose-dependently decreased breaking points and response rates in male and female rats. Gender differences were not observed. The results indicate that decreased food motivation may be a factor that contributes to the behavioral effects of PB administration.

Keywords: Pyridostigmine bromide; Gulf War; Progressive ratio schedule; Breaking point; Male and female rats

Pyridostigmine bromide (PB) is a quatenary ammonium compound that inhibits the hydrolysis of acetylcholine (ACh) by competitive reversible binding to acetylcholinesterase (AchE). PB may decrease nerve gas toxicity by occupying AChE binding sites (Dirnhuber et al., 1979; Wolthuis and van Wersch, 1984). Reportedly, PB was taken prophylactically during the Gulf War when there was a high risk of nerve gas exposure (Hoy et al., 2000a). PB exposure has been implicated in the development of Gulf War syndrome (Abou-Donia et al., 1996; Coker, 1996; Haley and Kurt, 1997; Haley et al., 1997a; Haley et al., 1997b). It should be noted, however, that soldiers may have been exposed to a host of other chemicals and/or stressors that may or may not have affected their health (Institute of Medicine, 1996; The Iowa Persian Gulf Study Group, 1997).

Previous research has shown that even small doses of PB can have serious behavioral effects. Wolthuis and van Wersch (Wolthuis and van Wersch, 1984) determined in rats that PB decreased two-way shuttle-box avoidance efficiency, decreased open-field locomotor activity, and produced a dose-dependent decrease in the number of correct steps in a hurdle-stepping task. In other studies, Liu et al. (Liu, 1991; Liu, 1992; Shih et al., 1991) observed that low doses of PB that did not produce any overt signs of toxicity decreased response rates maintained by a fixed-ratio (FR) schedule of reinforcement. We have recently reported that PB dose-dependently decreased locomotor activity in male and female rats, and more so in female rats (Hoy et al., 1999; Hoy et al., 2000a; Hoy et al., 2000b). We also showed that acute and repeated PB administration affected learning as it impeded response acquisition with immediate and delayed reinforcers (van Haaren et al., 1999; van Haaren et al., 2000a). In yet another study, we observed that PB dose-dependently decreased responding in male and female rats that was maintained by a fixed-interval (FI) 2-min schedule and a FR 50 schedule of reinforcement. Gender differences were not observed in this experiment, but FR rates were decreased by lower doses of PB than FI rates (van Haaren et al., 2000b).

Previous experiments have shown that PB administration decreases locomotor activity in male and female rats (Hoy et al., 1999; Hoy et al., 2000a; Hoy et al., 2000b), which could account for PB's effects on avoidance behavior and hurdle-stepping. It could also account for the effects of PB on food-maintained responding, but other variables may have also played a role. One of the variables that has not yet received experimental attention is the notion that PB administration may alter the reinforcing efficacy of food presentation. This

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question appears opportune as it has previously been shown that PB administration decreased voluntary water consumption in water-deprived rats without affecting locomotor activity (Liu, 1992). The present experiment was designed to investigate to what extent PB administration affects food motivation as measured with a progressive-ratio (PR) schedule of reinforcement. On PR schedules, the response requirement is systematically increased by (usually) a fixed number of responses after presentation of each reinforcer. The breaking point (in our case, the penultimate ratio that a subject completed before he/she failed to complete the ultimate ratio within a specific period of time) is the major dependent variable of interest. This breaking point can be assumed to reflect motivational variables inasmuch as it has been shown to vary systematically with increases in food deprivation and the volume and concentration of a liquid reinforcer (Hodos, 1961; Hodos and Kalman, 1963). The procedure has previously been used in our laboratories to show that there are no gender differences in food motivation in the absence of drug administration (van Hest et al., 1988). Male and female rats participated in the present experiment because previous studies have shown that the behavioral effects of drug administration (van Haaren, 1994; van Haaren and Anderson, 1994a; van Haaren and Anderson, 1994b; van Haaren et al., 1997), including PB (Hoy et al., 1999; Hoy et al., 2000a; Hoy et al., 2000b), may be a function of the subjects' gender.

1. Method

1.1. Subjects

Twelve male and twelve female Wistar–Hanover rats were obtained from a commercial supplier (Harlan Sprague–Dawley, Indianapolis, IN) when they were approximately 70-days-old. They were housed in same-sex groups of three under a reversed light–dark cycle (lights on 6:00 p.m.) and allowed free food and water for 1 week. Access to food was then limited for the remainder of the experiment (16 g/day per male rat and 12 g/day per female rat), while tap water remained continuously available. Male rats weighed an average of 369 g (range: 331–407 g) and female rats weighed an average of 245 g (range: 230–261 g) at the conclusion of the experiment. Experimental sessions were conducted during the subjects' dark hours between 9:00 a.m. and 3:00 p.m.

1.2. Apparatus

The experiment was conducted in six identical Coulbourn Instruments (Allentown, PA) modular rodent operant-conditioning chambers, that were 25 cm wide, 30 cm long and 29 cm high. The sides of each chamber were made of Plexiglas; the back wall and the intelligence panel were made of stainless steel. The floor consisted of 16 rods, spaced 2-cm apart (center to center). Two retractable rodent levers were located symmetrically to the side of the pellet tray, 6.3 cm from the floor of each chamber. When extended, the levers protruded 1.8 cm from the intelligence panel and required a force of more than 0.20 N to be operated. There were three stimulus lights directly above each lever and a house light was located 3 cm from the ceiling in the middle of the intelligence panel. The pellet tray was illuminated by a white light bulb during the delivery of a food pellet (Noyes, 45 mg purified rodent formula). Each experimental chamber was housed in an individual sound-attenuating, ventilated cabinet. The chambers were connected to an IBM-PC compatible microcomputer (GatorByte, Gainesville, FL) through a LabLinc interface (Coulbourn Instruments) located in the experimental room itself. Experimental contingencies and data acquisition procedures were programmed in L2T2 (Coulbourn Instruments).

1.3. Procedure

Lever pressing was first established according to a procedure that has been described in detail elsewhere (van Haaren, 1992). Subjects were then trained to respond on a PR 5 schedule of reinforcement. At the beginning of the session, the left lever was extended into the chamber and the stimulus lights above the lever as well as the house light were illuminated. The subjects were required to complete a PR 5 schedule to obtain the first food pellet. The response requirement was then increased by five responses after every food pellet presentation (i.e. PR 5, PR 10, PR 15, and so on) until the subject failed to complete the scheduled PR requirement within a 5-min period of time. Baseline sessions were conducted until the breaking point reached stability as indicated by visual inspection of day-to-day data plots. Individual stability was deemed to be present when there were no increasing or decreasing trends in breaking points across 10 sessions.

1.4. Drug preparation and drug administration

PB was obtained from Sigma (St. Louis, MO) and dissolved in distilled water. Different doses of PB (vehicle, 3, 10, or 30 mg/kg) were administered by gavage, 30 min prior to the start of an experimental session. These different doses of PB were selected because they had been used in previous experiments (e.g. Hoy et al., 1999; van Haaren et al., 2000b). Drug administration took place on Tuesdays and Fridays of each week, provided that baseline control rates were stable on Mondays and Thursdays. The different doses were administered at least twice (once in ascending and once in descending order). As is customary in our laboratory, additional doses were administered when there was a substantial difference in the behavioral effects of the initial two determinations. Frequently, the behavioral effects of intermediate doses need to be re-evaluated as their effects may change following exposure to higher doses of a drug.

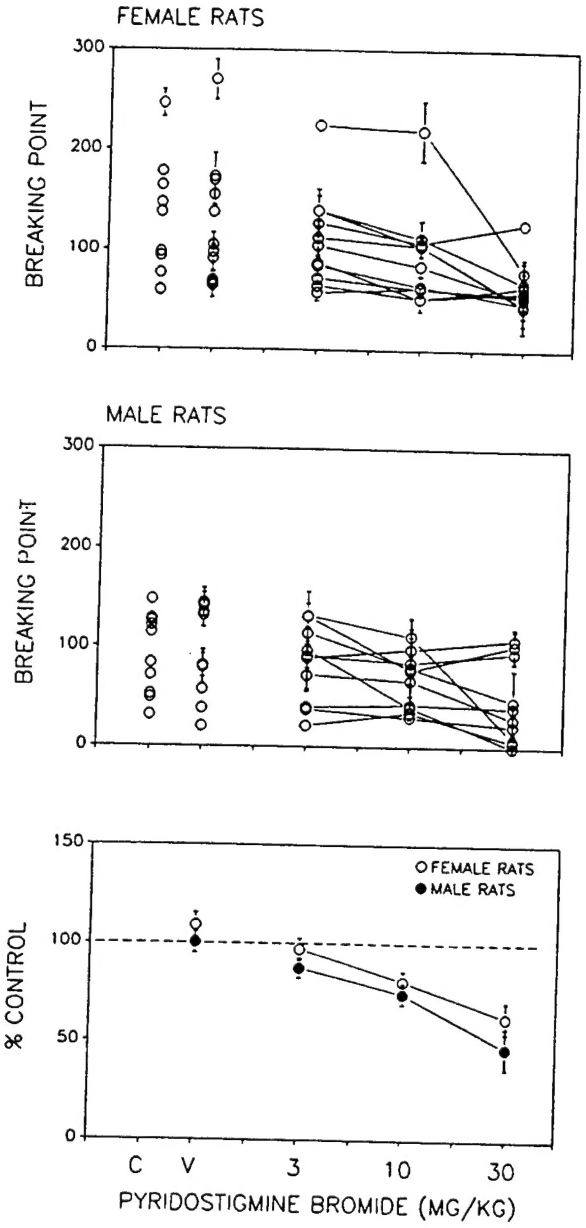

Fig. 1. Breaking points (failure to complete a specific ratio within 5 min, average ± 1 S.E.M.) as a function of the dose of PB in individual female rats (top panel) and individual male rats (middle panel). The data points depicted above 'C' refer to observations collected on the days immediately preceding those on which vehicle or drug was administered (control days). The bottom panel of the figure shows the data from the top and middle panels expressed as a percentage of these control values.

1.5. Statistical analyses

Analysis of variance including the factors Gender (male, female) and Dose (vehicle, 3, 10, or 30 mg/kg) was used to analyze breaking points and response rates. Duncan's new multiple range tests were used for post hoc comparisons. Significance levels were set at $P < .05$.

2. Results

Fig. 1 shows the breaking point as a function of the dose of PB in individual female rats (top panel) and individual male rats (middle panel). The data of one female rat and two male rats are not included because they failed to complete

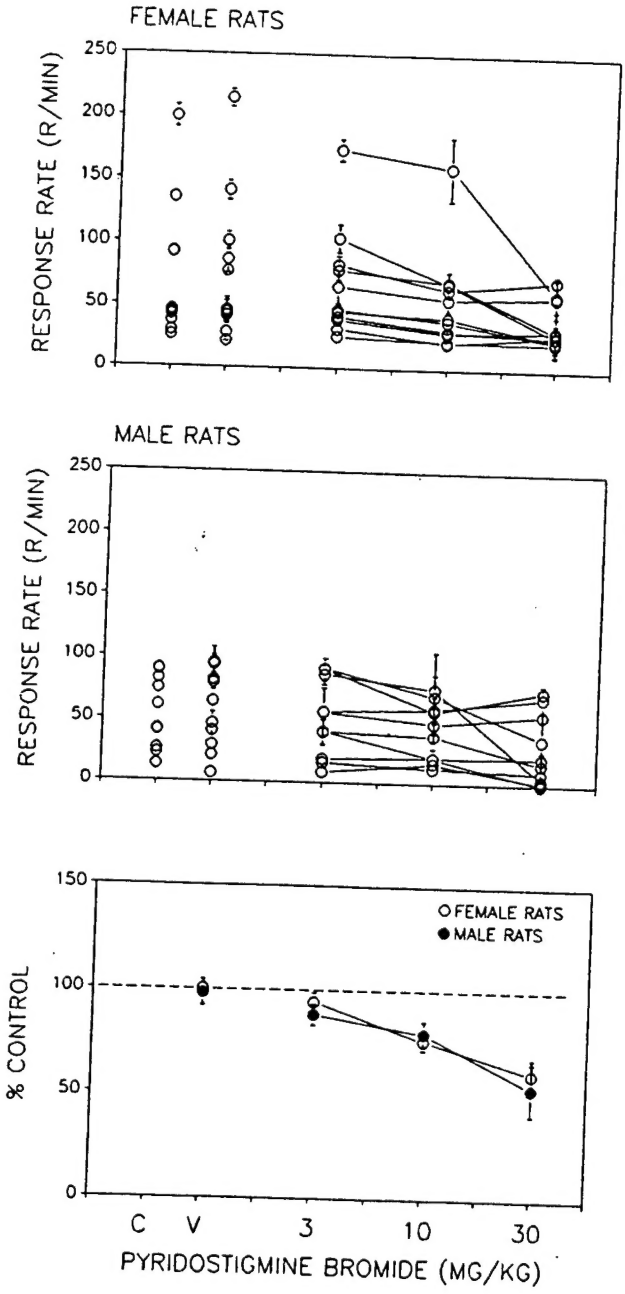

Fig. 2. Response rates (response per minute average ± 1 S.E.M.) as a function of the dose of PB in individual female rats (top panel) and individual male rats (middle panel). The data points depicted above 'C' refer to observations collected on the days immediately preceding those on which vehicle or drug was administered (control days). The bottom panel of the figure shows the data from the top and middle panels expressed as a percentage of these control values.

the assessment of the full dose–effect curve. Breaking points varied considerably within groups of male and female subjects. They were thus expressed as a function of nondrug control values (shown above 'C' in the top and middle panels of the figure) to facilitate comparisons. These data are shown in the bottom panel of the figure. Statistical analyses were performed on these normalized observations. Fig. 1 shows that breaking points decreased as a function of the dose of PB [$F(3,19)=27.95$, $P<.01$]. Differences between male and female rats were not observed [Gender, $F(1,19)=2.75$, n.s.] nor was there a significant interaction between drug administration and the gender of the experimental subjects [$F(3,19)=0.24$, n.s.]. Post hoc analyses, which combined the data for male and female rats across doses, showed that all three doses of PB decreased breaking points as compared to vehicle administration and that the differences between doses were also significant.

Fig. 2 shows response rates (responses per minute) as a function of the dose of PB in individual female rats (top panel) and individual male rats (middle panel). Response rates (which varied considerably within groups of male and female subjects) were also expressed as a function of nondrug control values (shown above 'C' in the top and middle panels of the figure) to facilitate comparisons. These data are shown in the bottom panel of the figure. As before, statistical analyses were performed on these normalized observations. Fig. 2 shows that response rates also decreased as a function of the dose of PB administration [PB, $F(3,19)=16.94$, $P<.01$]. Differences between male and female rats were not observed [Gender, $F(1,19)=0.32$, n.s.], nor was there an interaction between PB administration and the gender of the experimental subjects [$F(3,19)=0.28$, n.s.]. Post hoc analyses, which combined the data for male and female rats across doses, showed that, compared to vehicle administration, response rates were significantly lower after the administration of 10 and 30 mg/kg PB, but not after the administration of 3 mg/kg PB.

3. Discussion

The present experiment was designed to investigate whether or not PB administration would differentially affect responding maintained by a PR 5 schedule of reinforcement. Our results indicate that PB administration dose-dependently decreased breaking points and response rates maintained by a PR 5 schedule of reinforcement in male and female rats. The lowest dose of PB did not decrease response rates, but slightly altered food motivation as reflected by a small decrease in breaking points. Sex differences were not observed.

When drug administration decreases schedule-controlled responding, it can be due to a variety of factors. For instance, drug administration may affect motor activity making it more difficult for the subject to execute the operant response, or drug administration may increase responses incompatible with the operant response (such as locomotor activity). Alternatively, drug administration may alter the palatability of the reinforcer, or drug administration may induce nausea or such to decrease the efficacy of the reinforcer used to maintain the operant response. Or it may be that changes in motivation are a consequence of drug administration. The results of the present experiment suggest that the latter may be the case when subjects are injected with a low dose of PB. Our data indicate that breaking points were decreased by a lower dose of PB than response rates. Thus, it appears reasonable to suggest that PB administration may have altered the reinforcing efficacy of food presentation in experimental subjects that had limited access to food in the home cage. It should be noted, however, that this was a very small effect, which will have to be corroborated in other experiments employing other reinforcers (access to water, reinforcing brain stimulation). Our data argue against explanations that make reference to changes in motor function, such as an increase in the frequency of responses incompatible with the reinforced response. The lowest dose of PB decreased breaking points, but not response rates. This observation precludes an explanation in terms of an increase in incompatible responses, especially in view of the fact that previous studies conducted in our laboratory have indicated that PB administration tends to decrease locomotor activity (Hoy et al., 1999; Hoy et al., 2000a; Hoy et al., 2000b). Previous experiments have shown that PB administration may induce observable signs of cholinergic challenge following doses that exceed 20 mg/kg (Liu, 1992; Shih et al., 1991). In this context, to explain the differential effect of PB's low dose on breaking points and response rates in terms of drug-induced nausea or such appears an untenable position as well. In summary, these data and those of others who measured voluntary water consumption in water-deprived rats following PB administration support a role of PB in affecting motivational variables (cf. Liu, 1992).

Sex differences were not observed in the present experiment. As such these observations confirm those of other studies in which we also showed that PB administration decreases response rates maintained by different schedules of reinforcement, but that it does so equally in both male and female rats (van Haaren et al., 2000b). It should be noted, however, that we observed gender-dependent effects of PB administration when we studied locomotor activity in other experiments (Hoy et al., 1999). Apparently, PB's behavioral effects are different when evaluated against a baseline of spontaneous or unlearned behavior then when evaluated against a baseline of schedule-controlled or learned behavior. These observations again emphasize the importance of evaluating the behavioral effects of a drug under a variety of experimental procedures to appreciate its full range of behavioral effects.

The results of this experiment suggest that even a small dose of a compound that may have been used or administered at the time of the Gulf War can disrupt operant

performance. It remains to be determined to what extent the disruptive effects should be attributed to its central of peripheral actions. Studies in which peripheral cholinergic muscarinic receptors are blocked with the appropriate antagonists should shed further light on these issues (Liu, 1991), as might experiments in which subjects are stressed prior to or during drug exposure (Friedman et al., 1996). Such shall be the charge for investigators who want to further elucidate the behavioral effects of PB in future experiments.

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